ENGINEERING AN HIV RESISTANT IMMUNE SYSTEM

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Table of Contents

SUMMARY 5

ZUSAMMENFASSUNG 7

INTRODUCTION 9

1.1 The Human Immunodeficiency Virus 9
1.2 The HIV pandemic, impact and current treatment strategies 10
1.3 Highly active antiretroviral therapy (HAART) and its effect on HIV decline 11
1.4 HIV latency 12
1.5 HIV vaccines 15
1.6 HIV gene therapy 17

AIMS OF THE THESIS 21

RESEARCH ARTICLE 1 22

Optimization of critical hairpin features allows miRNA-based gene knock-down upon single-copy transduction 22
INTRODUCTION 24
RESULTS 27
DISCUSSION 44
MATERIALS AND METHODS 47
ACKNOWLEDGEMENTS 51
SUPPLEMENTARY MATERIAL 52

RESEARCH ARTICLE 2 59

Lentivector knock-down of CCR5 in hematopoietic stem cells confers 2 functional and persistent HIV-1 resistance in humanized mice 59

IMPORTANCE 61
INTRODUCTION 62
RESULTS 65
DISCUSSION 80
MATERIALS AND METHODS 85

ACKNOWLEDGEMENTS 88
SUPPLEMENTARY MATERIAL 89

VARIA 96

i) Original article *(In review)* 96
Discovery and characterization of an endogenous CXCR4 antagonist 96
ii) Original article *(In review)* 98
T Cell Engaging Bispecific Antibody Constructs Targeting HIV-1 Env-expressing Cells Show Significant Antiviral Efficacy 98
iii) Original article *(In preparation)* 100
Studying HIV-triggered EBV-associated lymphoproliferative diseases in a humanized mouse model 100
iv) Review article *(In preparation)* 108
Clinical applications of genetically modified hematopoietic stem cells 108
v) Review article *(Published: Progress in Neurobiology. 2011. Vol. 93. Pg.293-311)* 110
The chemokine receptor CCR5 in the central nervous system 110
vi) Review article *(Published: Pharmacology & Therapeutics. 2012. Vol. 133. Pg. 280-290.)* 111
Cardiovascular Pharmacogenetics 111
vii) Review article *(Published: European Journal of Cell Biology. 2013. Vol. 92. Pg. 229-236)* 113
Adipocyte and adipogenesis 113
viii) Project underway 115
Development of an *in vitro* and/or *in vivo* selection strategy for positive and negative selection of transduced $CD34^+$ progenitor cells 115
ix) Invention disclosures 120
University of Geneva, UNITEC: New design of micro RNAs (miRNAs) for gene therapy vectors 120
University of Geneva, UNITEC: *in vivo* cell selection via gene therapy and combination cytotoxic treatment 120

DISCUSSION 121

OUTLOOK 130

ACKNOWLEDGEMENTS 133

CURRICULUM VITAE 134

PUBLICATIONS 135

REFERENCES 137

SUMMARY

Gene therapy to cure HIV is now a reality and various clinical trials are underway. Since the development of humanized mouse models, studying human specific infectious disease such as HIV in the context of gene therapy has become more accessible, previously these types of studies were done in primate models using SIV. Humanized mice to study HIV gene therapy has therefore gained massive impetus over the last decade and there is a remarkable variety of strategies implemented thus far in pre-clinical humanized mouse studies. The work of this thesis focuses on an HIV gene therapy strategy where the therapeutic gene is a microRNA which eliminates expression of a critical host co-factor (CCR5 protein) required for HIV infection. The therapeutic microRNA is delivered to the target cells with an HIV-1 lentiviral vector.

One first aim was to develop a novel microRNA with superior gene knockdown capabilities. We looked at and modified various structural features of microRNAs such as lower stem lengths, upper stem configurations and thermodynamics of the upper stem to try and come up with a design that would improve processing of the microRNA by the enzymes DROSHA and DICER. Improved processing of microRNAs by these enzymes translates into more targeting strand being excised from the microRNA which is the effector of gene knockdown. The gene we aimed to knockdown in the initial studies was human CCR5 (a critical co-factor for HIV entry into target cells) and reporter gene GFP. The culmination of the experiments results in a new microRNA which we called mirGE and consisted of the following structural features: Lower stem length of 13 nucleotides, targeting strand carried on the 5' side of the microRNA, the 5’ end of the targeting strand is thermodynamically unstable compared to the 3’ end and has a wobble in the middle of this upper stem. We compared our mirGE to a commercially available microRNA, miR-30. mirGE proved superior in the following ways: amount of targeting strand made available for incorporation into RISC; ensuring the correct strand (targeting strand) is incorporation into RISC and we improved the precision of DROSHA cleavage from about 21% inaccuracy to 0.027%. We could achieve an efficiency of over 90% knockdown of CCR5 with

extremely low transduction rates, which proved sufficient to render cells resistant to infection with CCR5 tropic HIV.

Our second aim was to test this mirGE CCR5 knockdown lentivector in vivo in a humanized mouse model to determine whether we can transduce human hematopoietic progenitor cells ($CD34^+$ cells), whether these transduced cells expressing mirGE can engraft and give rise to mirGE expressing progeny cells in the periphery of the mice and whether the HIV target cells $CD4^+$ T cells in the mice are protected against HIV during chronic infection. We discovered that in order to inhibit HIV viral replication in the mice we had to achieve mirGE expression in >90% of HIV target cells in the periphery of the animals. This high frequency of transduced cells in the mice was achieved by sorting the $CD34^+$ cells transduced with the mirGE lentivector by flow cytometry. These purified mirGE expressing cells were transplanted into the mice which showed functional and persistent resistance to CCR5-tropic HIV for over 6 months and survival of human $CD4^+$ T cells despite HIV challenge.

ZUSAMMENFASSUNG

Gentherapie als Ansatz zur Heilung von HIV –infizierten Individuen ist nun Wirklichkeit geworden und verschiedene klinische Studien sind zurzeit im Gange. Seit der Entwicklung von humanisierten Mausmodellen, wurden gewisse Infektionskrankheiten die spezifisch für den Menschen sind, wie HIV, und deren Behandlung durch Gentherapie, einer umfassenderen Untersuchung zugänglich. Bisher mussten solche Untersuchungen in Primaten mit SIV durchgeführt werden. Das erforschen von HIV Gentherapien in humanisierten Mäusen hat deshalb einen massiven Auftrieb im letzten Jahrzehnt erlebt und es gibt eine bemerkenswerte Vielfalt an Strategien, die bisher in diesem präklinischen Model erprobt wurden. Der Focus dieser Arbeit ist eine HIV-Gentherapie-Strategie, wobei die Expression eines zelleigenen Gens (CCR5-Protein), welches kritisch ist für die Infektion durch HIV, gezielt durch eine spezifische microRNA eliminiert wird. Diese therapeutische microRNA wird durch einen HIV-1 lentiviralen Vektor in die Zielzellen eingebracht.

Unser erstes Ziel war es, eine neue microRNA mit überlegenen Knock-Down-Funktionen zu entwickeln. Wir haben verschiedene strukturelle Eigenschaften von microRNAs untersucht und modifiziert, wie zum Beispiel die untere Stem-Länge, die obere Stem-Konfiguration oder die thermodynamische Stabilität des oberen Stems, um die Verarbeitung der microRNA durch die Enzyme Drosha und DICER zu verbessern. Eine verbesserte Verarbeitung von microRNAs durch diese Enzyme führt zur Exzision von mehr Ziel-Strängen aus der microRNA, welche direkt für den Knock-Down verantwortlich sind. Das Zielgen in diesen Knock-Down-Studien war das menschliche CCR5 (ein wichtiger Ko-Faktor für den HIV-Eintritt in die Zielzellen) und das Reportergen GFP. Das Endresultat dieser Versuche war eine neue microRNA, welche wir mirGE bezeichnet haben und die folgenden Strukturmerkmale aufweist: Eine untere Stem-Länge von 13 Nukleotiden, eine Ziel-Sequenz am 5’-Ende der microRNA, ein 5'-Ende des Ziel-Strangs, welches thermodynamisch instabil ist im Vergleich zum 3'-Ende und eine Wobble-Sequenz besitz in der Mitte des oberen Stems. Wir haben unsere mirGE mit einer kommerziell erhältlichen microRNA, miR-30, verglichen. Die mirGE hat sich in den folgenden Arten und

Weisen als überlegen erwiesen: Mehr Ziel-Strang wurde produziert und stand zum Einbau in den RISC-Komplex zur Verfügung; Die Spezifität mit welcher der korrekte Strang (Ziel-Strang) in den RISC-Komplex eingebracht wurde war höher und eine verbesserte Präzision der Drosha Spaltung von etwa 21% Ungenauigkeit auf 0,027% konnte nachgewiesen werden. Wir könnten einen CCR5 knock-down von über 90% erreichen trotz einer niedrigen Transduktionseffizienz. Dieser Knock-Down erwies sich als ausreichend um Zellen resistent zu machen gegen eine Infektion mit CCR5 tropischem HIV.

Unser zweites Ziel war es, die mirGE in vivo in einem humanisierten Mausmodell zu testen, um zu bestimmen, ob wir humane hämatopoetische Vorläuferzellen ($CD34^+$ Zellen) transduzieren können, ob diese transduzierten Zellen, welche die mirGE exprimieren, in vivo anwachsen und zu mirGE exprimierenden Tochterzellen in den Mäusen führen und ob die HIV-Zielzellen, die $CD4^+$ T Zellen, in diesen Mäusen während chronischer Infektion von HIV geschützt bleiben. Wir haben entdeckt, dass die mirGE Expression in über 90% der HIV-Zielzellen im peripheren Blut vorhanden sein muss um die HIV-Virusreplikation in den Mäusen zu unterdrücken. Diese hohe Frequenz von mirGE transduzierten Zellen im Blut der Mäuse wurde erzielt durch eine durchflusszytometrische Selektion der $CD34^+$ Zellen, welche mit dem mirGE lentivector transduziert wurden. Diese aufgereinigte Population von mirGE exprimierenden Zellen wurde in Mäuse transplantiert, welche daraufhin eine funktionelle und anhaltende Resistenz gegen CCR5-tropisches HIV aufwiesen. Diese Resistenz hat über 6 Monate angedauert und hat das Überleben von menschlichen $CD4^+$ T Zellen ermöglicht, trotz HIV-Infektion.

INTRODUCTION

1.1 The Human Immunodeficiency Virus

The human immunodeficiency virus (HIV) is a lentivirus and can cause acquired immunodeficiency syndrome (AIDS). In humans AIDS results in the progressive failure of the immune system and people with AIDS suffer from constant opportunistic infections and cancers [1]. One of the most important aspects of the virus is its high rate of evolution or tendency to mutate at a genetic level rather quickly. This is the major hurdle that makes effective treatment of the disease so difficult, since it has been estimated that for HIV-1, single mutations that could render a particular drug ineffective can occur on a daily basis, making the use of multiple drugs obligatory [2].

HIV belongs to the *Retroviridiae* family of viruses, the main characteristics of these viruses is that they are enveloped with a single stranded RNA genome. The RNA genome is reverse transcribed into DNA which is stably integrated into the genome of the host or target cell. This proviral genome serves as a template for the production of new viral RNAs, proteins and eventually the assembly of progeny virions. More specifically, HIV is part of the *lentivirus* subfamily, the hallmarks of lentiviruses is that their genomes are more complex than other retroviruses and lentiviruses result in a slow and degenerative disease which can last decades, compared to other retroviruses such as oncoretroviruses which cause rapidly developing tumours. The HIV genome can be split into two parts, the non-coding or cis-acting sequences and the coding sequences. The non-coding sequences are responsible for replication, integration and expression of proviral sequences and include the 5' cap, the R (repeated) region which is present in the 5' LTR as well as the 3' LTR and contains the TAR sequence which allows binding of TAT, the U5 sequence which contains the att sites allowing for proviral integration, the PBS site which binds tRNA which serves as a primer for reverse transcription, the Psi element which is the signal for encapsidation of viral RNA into the viral particle, the central and

distal polypurine tracts which are primers for formation of plus strand DNA, the Rev-responsive element which is recognised by Rev and facilitates export of viral mRNAs to the cytoplasm and finally the U3 region again containing sequences required for DNA integration. The coding sequences include the LTRs which contain regions that bind host transcription factors, the Gag and Env genes which code for the structural proteins of the virus such as the matrix, capsid, nucleocapsid and gp160 envelope protein respectively. The Pol gene codes for viral enzymes such as protease, reverse transcriptase and integrase. Finally, there are 6 regulatory or accessory genes; Vif, Vpr, Vpu, Nef, Tat and Rev which together allow the virus to infect cells and replicate [3].

1.2 The HIV pandemic, impact and current treatment strategies

More than 39 million people have died worldwide in the HIV pandemic from AIDS (http://www.unaids.org/sites/default/files/media_asset/01_PeoplelivingwithHIV.pdf). South Africa with ~50 million habitants is one of the worst affected regions and is faced with a major HIV/AIDS burden with an estimated prevalence of 17.9% (~6.1 million HIV-positive people); the estimated number of AIDS orphans mounts to 2.5 million AIDS orphans (www.UNAIDS.org 2012). South Africa's challenge is to address various issues surrounding the diseased including prevention, treatment and education. Currently, the only effective treatment for HIV-associated immunodeficiency is highly active antiretroviral therapy (HAART), resulting in an impressive decrease in HIV-associated morbidity and mortality [4,5]. However, widespread implementation of HAART has revealed a large number of problems, such as access to HIV clinics, emergence of resistant viral strains in case of non-adherence, and the side effects of lifelong use. Despite HAART which can allow patients to live a relatively normal life with HIV, there is still no cure. There are three main treatment strategies i) vaccines, ii) development of drugs to induce and purge the latent reservoir which would be used in combination with HAART and iii) gene therapy. All three approaches are being explored to provide a sterile cure (eliminating any trace

of viremia) or to provide a functional cure (persistence of a low but stable viremia without any impact on the immune system).

1.3 Highly active antiretroviral therapy (HAART) and its effect on HIV decline

HIV can now be considered as a chronic disease that can be managed due to the advances in HAART over the last three decades. HAART combines conventionally three drugs, i.e., two drugs belonging to the family of the nucleoside reverse transcriptase inhibitors (NRTIs) and a 3rd one which is either a non-nucleoside reverse transcriptase inhibitor(NNRTI), a protease inhibitor (PI) or an integrase inhibitor (INSTI). Using a combination of drugs ensures that multiple steps in the HIV life cycle are targeted simultaneously, minimizing the risk of emergence of resistant strains. HAART is able to decrease viremia to below detection limits. Once a patient receives HAART, the decline in viral load can be described in four stages, where in each stage there is a smaller possibility of eliminating infected cells and thus a slower decline in viremia in each subsequent stage which is linked to the increased half-life of various distinct cells types involved in HIV infection.

Stage 1: Occurs within 2 weeks from the start of treatment: There is a sharp decline in peripheral blood viremia which has been attributed to the elimination of productively infected cells due to the drugs preventing new rounds of infection; in parallel we note a recovery of $CD4^+$ T cells in the blood. The strikingly rapid decline in blood viremia during stage one is directly related to the relatively short half-lives of free virus in the blood (~6 hours) and productively infected cells (<2 days) [6,7]. **Stage 2; Occurs much slower than stage 1:** During this phase the continued decline in viremia has been accounted to the elimination of long lived infected cells, such as macrophages and dendritic cells as well as partially activated $CD4^+$ T cells. The increased half-life of infected macrophages compared to infected $CD4^+$ T cells is because infected macrophages are less susceptible to the cytopathic effects of HIV [8]. Partially activated $CD4^+$ T cells have an increased half-life compared to HIV-infected fully activated $CD4^+$ T cells which is the most likely reason for the slower viremia decline in this stage [8]. Indeed the findings

related to resting memory $CD4^+$ T cells (the first definition of an HIV latent reservoir, [9] showed that these cells have a half-life of over 40 months but when activated can produce replication competent virus. [10,11]. It is likely that when such resting $CD4^+$ T cells are activated and large amount of viral replication occurs that the cells do not survive [12]. Therefore it has been suggested that the pool of latently infected memory $CD4^+$ T cells is propagated when these cells are infected in the declining phase of activation and progression to the active replication phase is blocked [13]. It has been suggested that a mechanism that allows for the propagation of latently infected memory $CD4^+$ T cells is low level homeostatic proliferation of this pool of cells caused by the presence of antigens and interleukin-7 [14]. **Stage 3 and 4:** These stages have been described as consisting of a pool of central memory (T_{CM}) and transitional memory (T_{TM}) $CD4^+$ T cells which can persist for decades depending on patient $CD4^+$ T-cell counts and how early HAART was started [14]. In fact the size of the reservoir depends on how early HAART is started, but will otherwise not affect disease outcome. To conclude, HAART is very effective and provides patients around the world with a strategy to live with the disease. However, the persistence of the virus despite treatment requires lifelong adherence which is the main draw-back of HAART therapy.

1.4 HIV latency

As outlined above, HAART effectively reduces blood viremia and productively infected cells but has no effect on infected cells with a long half-life and which do not enter into a phase of productive infection defined as a state where the HIV promoter is actively transcribing genomic viral RNA and producing progeny virus. Instead cells can be infected and the HIV promoter can exist in an inactive state resulting in no transcription of viral RNAs and no production of progeny virus. Latency is a dynamic process whereby HIV is able to secure its long-term and almost sustainable survival in the host. Latency is one of the ways but not the major way that HIV escapes immune surveillance; it is also a way that HIV preserves its presence in the host by avoiding the cytopathic effects of producing progeny virus [15] [16] [17].

i) Establishment of latency: There are two current understandings of how latency results in viral persistence despite HAART treatment. The first is linked to the normal life cycle of $CD4^+$ T cells which upon exposure to antigen the resting $CD4^+$ T cells become activated, proliferate and differentiate producing effector $CD4^+$ T cells, some of which do not die but return to the resting stage and become long lived memory cells primed for an improved response upon re-exposure to the antigen. HIV usually infects activated $CD4^+$ T cells, once the proviral DNA has integrated into the genome of the cell it comes under the control of host transcriptional activation and deactivation where the cellular transcription factor nuclear factor-$_K$B (NF_KB) and specificity protein 1 (Sp1) initiate transcription of viral accessory proteins [18]. Once the HIV Tat protein (viral transactivator of transcription) reaches a critical level in the cell, rate of viral transcription is significantly increased resulting in virus particle production and cell death [18]. Many newly infected $CD4^+$ T cells die because of the cytotoxic effect of the virus as well as host immune responses. However, a proportion of these cells despite being infected by HIV survive and return to a resting memory state, and it is during this moment that host transcription factor dependant HIV transcription falls silent in a cell that is now programmed to survive for an extended period of time [16]. It is still not completely clear whether HIV can infect and establish latency in resting $CD4^+$ T cells or whether it is by infection of activated $CD4^+$ T cells which survive and revert to a resting state as described above [18] – in any case, latent HIV is found mostly in long-lived memory $CD4^+$ T cells [14]. These latently infected cells (about 1 per $1x10^6$ cells) contain integrated but transcriptionally inactive proviral DNA and are the reason why patients despite having access to HAART cannot be cured of the disease. The second understanding is that HAART in tissues such as the gut-associated lymphoid tissue and central nervous system drug penetration is not optimal and low level HIV replication continues in these tissues which results in establishment and continuous renewal of a substantial reservoir of latently infected cells [19,20].

ii) Maintenance of latency: There are various mechanisms which have been elucidated that explain how HIV latency is maintained. HIV-1 usually integrates in gene regions that are actively

transcribed; this it seems somewhat counterintuitive for the existence/maintenance of latent provirus; this phenomenon is explained by promoter exclusion and convergent transcription: promoter exclusion results in HIV expression being repressed by transcription factors being displaced from the HIV-1 LTR by read-through of the host RNA polymerase II since the proviral DNA is in the same orientation as the host gene [21]; convergent transcription results in collisions of the host RNA polymerase II due to the HIV-1 provirus having integrated in the opposite orientation as compared to the host gene [22]. In addition, the chromatin structure of genes determines whether the particular genes are accessible to the various transcription factors. Posttranslational modifications such as acetylation and methylation of histones contribute to changes in chromatin structure resulting in either more repressive or active chromatin states. The role of histone deacytelases (HDACs) in maintaining the transcriptionally silent state of the HIV-1 genome in latently infected cells has been studied in detail and they regulate this process in various ways [18]. HIV latency is directly regulated by HDACs through deacytelation of histone lysine residues which limits the access of transcription factors to the DNA and indirectly by non-histone protein modification or deacytelation of the RelA subunit of NF-αB for example [18]. This repressive state also recruits further histone-modifying complexes that contribute to the silent state [23]. In the HIV latent state HDACs are brought proximal to the integrated HIV proviral DNA LTR by various transcription factors of the cell such as retinoblastoma family protein 2 (RBF2), nuclear factor-KB p50 (NF-KB), c-promoter-binding factor 1 (CBF-1), specificity protein 1 (Sp1) and Ying-Yang 1 (YY1) [18]. Nuc-1, the repressor nucleosome which is positioned downstream of the HIV transcriptional start site during the latent stage requires for its positioning the BRG1/brm-associated factor (BAF) complex which is associated with HDAC1 & HDAC2. Furthermore, the methyl binding protein (MBD2) detects methylated DNA proximal to nuc-1 and recruits the nucleosome remodelling and histone deacetylation complex (NURD) which is also associated with HDAC1 and HDAC2 [18]. HDAC3 also deacytelates NF-KB (p65-p50) and negatively regulates its full activation.

iii) Activation of latency: Activation of latently infected cells which would make purging of these cells possible is considered as the key to a sterilizing cure for HIV. Patients on HAART, as discussed, have latently infected cells surviving despite long-term treatment. The strategy is to bring latently infected cells of HAART treated patients out of this silent state which would make these cells visible to the immune system and thus being killed by HIV-specific CTLs or NK cells, or would die due to the cytopathic effects of viral replication. The ongoing HAART would prevent spreading infection. Various strategies to block the factors which are involved in repression of HIV provirus have been proposed. One example is the NF-$_K$B p50/p50 homodimer and HDAC 1 [24]. Indeed studies have used various HDAC inhibitors for this purpose; valproic acid [25] which was reported to reduce the number of HIV-1 infected resting cells, however this was not confirmed in later studies [26,27]. A potent class 1 HDAC inhibitor, suberoylanilide hydroxamic acid or SAHA (Vorinostat) induces HIV-1 transcription in resting $CD4^+$ T cells in patients on HAART [23]. One of the major challenges is to accurately define the size of the latent reservoir because this would allow one to define whether the reactivation and purging is leading to a decrease in the size of the reservoir. It is very difficult to estimate the size of the latent reservoir in a patient and this is currently done through the viral outgrowth assay which can provides an estimate of the minimum number of latently infected cells that should be eliminated, or with various PCR assay detecting HIV DNA [28]. The main problem with the viral outgrowth assay is the labour intensiveness and high costs, while the PCR based assays cannot distinguish between replication-competent and defective viral DNAs - this results in the PCR methods indicating infected cell frequencies up to 2 fold higher compared to viral outgrowth assays [28].

1.5 HIV vaccines

After decades of work, there is still no efficacious vaccine for HIV [29]. A distinction must be made between prophylactic and therapeutic vaccines. Notably, there are dozens of examples of various approaches and multiple clinical trials at different stages being conducted [30,31]. Notably,

despite a quarter of a century's research there is still no evidence that an effective prophylactic vaccine is possible [30].

Prophylactic vaccines: *A careful exposure of an attenuated form of for example a virus prior to the onset of an infection which will induce the immune system to produce specific antibodies against the particular virus.*

There has been only one phase III vaccine trial for HIV. The "Thai trial" showed moderate 31.2% efficacy, that is fewer infections over 42 months [32]. This vaccine called RV144 was based on a prime-boost strategy, i.e., prime with ALVAC which is a canarypox-vector (based on an inert canarypox virus which cannot replicate in humans) containing three genetically engineered HIV genes; Env, Gag and Pol, and then a boost with AIDSVAX which is a genetically engineered HIV envelope gp120 glycoprotein. The success of this trial was correlated with the induction of non-neutralizing IgG antibodies binding to the envelope V1V2 loop [33]. Careful analysis of immune-correlates of the Thai trial revealed that Env binding IgA antibodies lessen the anti-HIV effect of the protecting V1V2 binding IgG antibodies [33]. This is a very positive indication that the foundation to a robust HIV vaccine has been laid, however this level of efficacy (32%) is not an acceptable success rate allowing for routine clinical application of the vaccine.

Therapeutic vaccines: *These are treatment vaccines, they are vaccines designed to treat a person already infected with for example a virus and aim to boost the immune system to help control the infection.*

There is now a lot of attention being focused on the development of a therapeutic vaccine for HIV. The main advantage of therapeutic vaccines is that it is being used on HIV infected individuals, this allows for a much quicker and more cost effective determination of how effective the vaccine is, which specific factors are resulting in the protection and whether the vaccination can complement traditional HAART. This then allows for a real-time assessment that depending on the level of efficacy of the vaccine HAART could be scaled down, simplified or even interrupted [34]. There are two main arms of therapeutic vaccine research, developing

vaccines that induce a humoral immune response and vaccines that induce cellular immunity. An example of a current therapeutic vaccine intended to induce a humoral response is VAC-3S. In HIV infected patients the HIV gp41 3S domain induces expression of NKp44L on uninfected $CD4^+$ T–cells which makes these cells more susceptible to lysis by $NKp44^+$ activated NK cells. VAC-3S peptide immunization should result in the production of anti-gp41-3S antibodies which block expression of NKp44L on $CD4^+$ T cells and protect these cells against activated NK cell lysis [34-36]. The HVTN 502 and 503 vaccines were designed to induce a cellular response to HIV and are based on a replication competent simian CMV vector with expresses engineered SIV Gag, Tat, Rev, Nef, Env and Pol [37-39]. This vaccine induces protective effector memory $CD8^+$ T cells which were restricted by MHCII antigens in primate models with some promising results where 50% of vaccinated monkeys were protected [37-39]. The main issue with this strategy is its transferability to humans, one aspect using an appropriate vector. Finally, approaches are being investigated which try to define more specifically relevant epitopes using bioinformatics technology to design mosaic or conserved antigens to be used as vaccines [40-42]. The aim here being is to improve the extent of both humoral and cellular immune responses.

1.6 HIV gene therapy

Gene engineering HIV resistant T cells got a marked impetus with the first HIV-infected person supposedly cured from HIV [43-45]: the patient received bone marrow from a CCR5Δ32 homozygous donor to treat acute myeloid leukemia, which eventually led to the patient undergoing a complete genotype shift from heterozygous to homozygous CCR5Δ32. Even though the patient stopped HAART before transplantation, he did not experience a viral rebound [45] [43]. CCR5 is the co-receptor of HIV for cell entry [46-50]; its lack renders cells resistant to HIV. This anecdotal case report suggests that stem/progenitor cell-based gene therapies targeting host and/or HIV genes is a promising strategy for cure of HIV. More recently it was reported that an HIV patient who developed anaplastic large cell lymphoma underwent a similar procedure and received donor CCR5Δ32 (homozygous) stem cells. The tropism of HIV in

this patient was determined prior to the transplantation with CCR5 deficient stem cells and confirmed as predominantly CCR5 tropic. HAART was discontinued prior to the transplantation and had to be resumed 3 weeks after the transplantation due to a viral rebound of up to 93'390 HIV RNA copies/ml. Tropism analysis at this point revealed that there was predominantly X4 tropic virus present, HAART successfully supressed the viral load again. However, the T-cell lymphoma relapsed and HAART was again discontinued, two weeks prior to the patient's death viral load was at 7'582'496 HIV RNA copies/ml. It was clear that there was a shift from predominantly R5 tropic HIC to X4 tropic HIV in this patient most likely due to the transplantation of CCR5 deficient stem cells [51]. There is also reference to other patients who had experienced viral rebound despite transplantation with CCR5 deficient stem cells [52]. This evidence indicates that targeting only CCR5 or only other HIV targets in a gene therapy approach to cure HIV might in some cases not be sufficient to prevent viral rebound due to tropism shift. It is important that further targets be identified and used in conjunction with CCR5.

The specificity of HIV to infect humans means that only sophisticated models such as monkeys or humanized mice are appropriate for studying gene therapies against HIV. Humanized mice are generated by the transplantation of human $CD34^+$ cells into immunocompromised mice – these cells engraft and build a lymphoid system of human origin, susceptible to HIV [53]. Several different humanized mouse models (NSG, BLT, NSG-BLT, NOD-RAG1-/-IL2rγ-/-) generated by manipulation and transplantation of human $CD34^+$ progenitors have been used to investigate the gene engineering modality, by silencing and/or modulating host and viral co-factors. Gene transfer was done using mainly lentiviral vectors encoding i) shRNAs against various viral or host cell targets, ii) broadly neutralizing antibodies, iii) HIV-specific T cell receptor, iv) TRIM5α isoforms, v) TAR decoys as well as using nucleofection of targeted zinc-finger nucleases targeting CCR5 [54 55 56 57 58 59]. The success to inhibit HIV replication varied greatly; in particular, partial viral load inhibition *in vivo* was demonstrated by gene engineering $CD34^+$ cells with a CCR5 targeting zinc finger nuclease, an HIV specific TCR or a broadly neutralizing antibody

[54,57,58]. Also by gene engineering of $CD4^+$ T cells with a CXCR4 targeting zinc finger or knockdown/overexpression of LEDGF/LEGDF mutants [60,61], which provided inhibition for relatively short periods of infection (7 - 56 days at the most). Several publications restricted their efforts to present the success of gene engineering HIV resistant cells in humanized mice with *ex vivo* experimentations with purified gene engineered cells such as splenocytes [55,56,59]. [62], showed that enrichment of anti-HIV transduced cells *in vivo,* however no *in vivo* infection data is shown. In this work transduced cells express the $MGMT^{P140K}$ drug resistance gene alongside with various anti-HIV genes, the $MGMT^{P140K}$ expression provides cells with resistance to combination treatment with bischloroethylnitrosourea (BCNU) and O6-benzylguanine (O6-BG) [62]. Thus, the studies done in humanized mice reveals the potential of gene engineering HIV resistant cells but none targeting cellular or viral factors resulted in cure definitive cure of HIV thus far.

One of the most commonly used tools for anti-HIV gene therapy is RNA interference where appropriate target genes are silenced by expression of shRNAs. Intriguingly, the less widely used cousin of the shRNA, the pri-miRNA mimic or miRNA has only rarely been tested for gene engineering. shRNA is considered more powerful than miRNAs, but more toxic and the use of shRNA may destabilize cellular homeostasis. miRNAs mimicking more closely natural pri-miRNAs have far less potential to result in those adverse effects [63-68]. miRNAs can be expressed using inducible or tissue specific RNA pol II promoters, while shRNAs in most cases are expressed using RNA pol III promoters which could be linked to destabilization of cellular homeostasis [64]. Thus, miRNA may be better suited for doing gene therapy provided an efficient knockdown of the gene of interest.

Despite the huge variation in techniques employed thus far, one major hurdle still has not been addressed which is common to all of the above mentioned studies. The transplantation of chimeric populations of gene engineered and non-gene engineered $CD34^+$ cells instead of a homogeneous population of gene engineered cells that is the most likely reason for the

continuing loss of $CD4^+$ T cells when targeting viral or cellular host factors by shRNA in clinical trials as well as in humanized mice.

AIMS OF THE THESIS

Provide *in vitro* and *in vivo* data as the foundation to develop a gene therapy cure for HIV in patients involving autologous bone marrow transplantation of anti-HIV gene engineered hematopoietic progenitor cells. Notably, HIV is dependent on critical host factors for its replication; one critical factor is the chemokine receptor CCR5 which in concert with CD4 constitutes the HIV's entry receptor complex for the majority of HIV strains. This gene engineering approach requires a highly efficient CCR5 targeting "RNA interference" miRNA without off-target effects that is safe for the clinical setting. We attempted to design such a CCR5 targeting miRNA system which we called mirGE (mir-Geneva). mirGE was specifically designed to improve upon the suboptimal knockdown efficiency achieved with an existing widely published miRNA system based on miR-30. I set out to i) test various designs of mirGE which would provide insights into what structural features of a miRNA are most important for improving efficiency of recognition and processing of miRNAs to achieve the most efficient target gene knockdown. ii) Studying the efficacy of mirGE to protect protect cells against multiple rounds of HIV infection *in vitro* and to determine whether this strategy can protect NOD SCID gamma (NSG) humanized mice against HIV infection *in vivo* and *ex vivo*. iii) Providing the proof of concept that future *in vivo* or *ex vitro* selection strategies would be required for the success of this type of HIV gene therapy approach since we hypothesise that in order to have a significant effect on HIV viral replication, the majority of HIV target cells should be anti-HIV gene engineered.

The two original papers that follow entitled **"Optimization of critical hairpin features allows miRNA-based gene knock-down upon single-copy transduction"** *(Published 2014: Molecular Therapy Nucleic Acids (2014)* ***3****, e207; doi:10.1038/mtna.2014.58)* and **"Lentivector knock-down of CCR5 in hematopoietic stem cells confers functional and persistent HIV-resistance in humanized mice"** *(In review)* are the main focus of this thesis.

RESEARCH ARTICLE 1

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Optimization of critical hairpin features allows miRNA-based gene knock-down upon single-copy transduction

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Gene knock-down using miRNA-based vector constructs is likely to become a prominent gene therapy approach. It was the aim of this study to improve the efficiency of gene knock-down through optimizing the structure of miRNA mimics. Knock-down of two target genes was analyzed: CCR5 and GFP. We describe here a novel and optimized miRNA mimic design called mirGE comprising a lower stem length of 13 base pairs, positioning of the targeting strand on the 5' side of the miRNA, together with nucleotide mismatches in upper stem positions 1 and 12 placed on the passenger strand. Our mirGE proved superior to miR-30 in four aspects: yield of targeting strand incorporation into RISC; incorporation into RISC of correct targeting strand; precision of cleavage by Drosha and ratio of targeting strand over passenger strand. A triple mirGE hairpin cassette targeting CCR5 was constructed. It allowed CCR5 knock-down with an efficiency of over 90% upon single copy transduction. Importantly, single copy expression of this construct rendered transduced target cells, including primary human macrophages, resistant to infection with a CCR5-tropic strain of HIV. Our results provide new insights for a better

knock-down efficiency of constructs containing miRNA. Our results also provide the proof-of-principle that cells can be rendered HIV-resistant through single copy vector transduction, rendering this approach more compatible with clinical applications.

INTRODUCTION

Micro RNAs (miRNAs) are naturally occurring, non-coding small RNAs, which regulate the expression of target genes [69], by degradation of their mRNA and/or block of their translation. The generation and processing of miRNA from miRNA genes follows a defined pattern [70]. Briefly, miRNAs are typically transcribed by RNA polymerase II as a primary miRNA (pri-miRNA) of several hundred nucleotides comprising a ~70 bp stem-loop structure. The stem-loop structure is then cleaved from the pri-miRNA by a "microprocessor complex" formed by the RNAse III enzyme Drosha and its subunit protein DGCR8, generating the precursor miRNA (pre-miRNA), which is similar in structure to shRNAs (short hairpin RNA) [71,72]. The pre-miRNAs are further processed in the cytoplasm by the endoribonuclease Dicer, which removes the loop of the hairpin, yielding a miRNA duplex of approximately 22 base pairs. The antisense strand (targeting strand in this paper) of the miRNA duplex will be integrated into the RNA-induced silencing complex (RISC), where it blocks translation through interaction with its target mRNA.

The elegance and efficiency of RNA interference has rapidly led to knock-down vectors designed from naturally occurring miRNAs. A first generation of lentivectors directly expressed shRNAs as a simple stem loop structure with no flanking sequences. When transcribed, they immediately form a thermodynamically stable stem loop and are directly exported to the cytoplasm where they are processed by DICER bypassing Drosha. In order to express shRNAs without flanking sequences, H1 [73], or U6 [74], RNA Pol III promoters or snRNA U1 Pol II promoters [68], were initially used. However, these promoters suffer from i) their constitutive expression and ii) potential toxicity due to competition of the artificial shRNA with endogenous miRNAs and an eventual subsequent saturation of the RNAi machinery, specifically the karyopherin exportin-5 [63,65]. Also, overexpression of shRNAs may stimulate the innate immune system through activation of the RNA-dependent protein kinase/interferon response [66]. On the other hand, miRNA mimics are compatible with expression from RNA pol II tissue specific or inducible

promoters and produce less processed antisense/targeting strand RNA, which if in excess can lead to cellular toxicity [64]. Thus, constructs reproducing natural miRNA synthesis and processing for the purpose of gene knock-down could be preferred to shRNA constructs. Indeed, artificial pri-miRNA mimics have been used for silencing a variety of target genes [67,75-77]. Most of these constructs were based on a naturally occurring miRNA miR-30 backbone with various flanking region, stem and loop modifications [67,69,75-83]. A similar version of this miR-30-based miRNA mimic is also commercially available (GIPZ & TRIPZ shRNAmir lentivector expression systems, Open Biosystems).

Lentiviral vectors containing either shRNA or miRNA are very promising tools for gene therapy involving gene repression. RNA interference as a tool for gene therapy has been explored *in vitro* and *in vivo* using both shRNA and miRNA mimics. Focusing on miRNA in lentivectors, the main targets involved were HIV [84,85], Hepatitis B virus [86], cancer [87], Alzheimer disease [88], only to mention a few. But the standards for such vector systems are high since they should achieve the required knock-down effect (ideally at a single transgene integration level) without affecting normal cell functions. Indeed, multiple transgene insertions into the host cell genome resulting from high vector-mediated transduction rates can increase the risk of alteration of functionally relevant parts of the genome and in particular increase the risk of oncogenesis [65,89].

In this study, we optimized miRNA-mimic design by adjusting complementary sequences and stem lengths (mirGE). We then chose the most potent variant, called mirGE herein after, and compared it to the original miR-30 in its efficiency to knock-down the CCR5 HIV co-receptor. Our mirGE proved largely more potent than miR-30. In particular, high-throughput sequencing revealed that mirGE is superior to miR-30 in four aspects: yield of targeting strand incorporation into RISC; incorporation into RISC of correct targeting strand; precision of cleavage by Drosha and ratio of targeting strand over passenger strand.

We increased mirGE efficiency by expressing it as a multiple hairpin structure and found that a triple hairpin anti-CCR5 mirGE down-regulates CCR5 expression by more than 90% with a

single copy of lentivector. This level of CCR5 knock-down efficiency by a single copy of anti-CCR5 lentivector protected HeLa cells as well as primary human macrophages against *in vitro* infection with a CCR5-tropic HIV strain . To the best of our knowledge, this is the most efficient miRNA-based lentivector described so far and the first demonstration that miRNA-based CCR5 knock-down by a single copy of a lentivector can confer intra-cellular immunization against HIV.

Our results thus pave the way towards an efficient yet clinically compatible gene therapy approach aimed at generating HIV-resistant immune cells in human patients.

RESULTS

Effect of miRNA hairpin features on green fluorescent protein knockdown.

To test miRNA hairpin features critical for efficient gene knock-down, we constructed a lentivector containing two independent expression units (**Supplementary Fig. S1**). One unit is expressing, under the control of the human Ubiquitin promoter, an mRNA containing the human microsomal glutathione S-transferase 2 (MGST2) gene ORF followed by a variable miRNA structure targeting the GFP living color. The MGST2 gene was used as spacer as we initially observed that repression activity was dramatically reduced when miRNAs were placed immediately after the transcription start site (data not shown). A similar finding has been reported that absence of intervening sequence between the 5' end of the mRNA and miRNA hairpin was detrimental to knock-down activity [67]. The other unit is expressing the mCherry living color under the control of the human PGK promoter. This dual cassette design allowed us to reliably follow transduced cells expressing constant amounts of red mCherry protein, while green GFP protein expression was down-regulated by the miRNA.

We then transduced a clone of HeLa cells stably expressing GFP (4.5 cells) [90], with lentivectors containing seven versions of a composite miRNA backbone containing the same 22 nt segment targeting the GFP living color (**Fig. 1**). This miRNA backbone contains the miR-16 lower stem as well as flanking regions, the target-specific stem containing a wooble at position +12 (as in miR-16) and the miR-30 loop. We kept the miR-30 loop since the terminal loop seems to be dispensable for pri-miRNA processing [72]. Variations on this backbone were made in order to test for two features, i.e. the length of the lower stem and the side of the mismatch on the targeting strand. D13M5 refers to a 13 bp long lower stem and a single nucleotide mismatch at the 5' end of the targeting strand, and is thus analogous to miR-16 [72], in terms of lower stem length and targeting strand mismatch orientation. Other hairpin designs follow the same numbering code, where the number after D represents the length of the lower stem and the number after M represents the mismatched end of the targeting strand. GFP-positive 4.5 cells

were transduced with similar MOIs of each vector and the percentage of GFP-negative cells generated within the total of mCherry-positive transduced cells was analyzed by flow cytometry (**Fig. 1b**). Percentages were then plotted for statistical analysis (**Fig. 1c**). The most obvious observation is that the length of the lower stem is critical for overall efficiency, as hairpins with 13 or 15 nucleotides in their lower stem were the most potent at generating GFP-negative cells. Longer (D17) or shorter (D11, D9) stem lengths show negligible effect on GFP down-regulation. Such an impact of lower stem length on knock-down efficiency was reported previously [77], and is likely due to the generation by Drosha processing of hairpins with lengths that are inappropriate for further steps in RISC processing [91]. Indeed, it was shown that the Drosha cleavage site is determined mostly by the junction between the flanking sequences and the lower stem and that cleavage occurs ~11bp (when counting on the bottom strand, i.e. 13 bp on the top strand as we number in this paper) up from this junction [72]. Comparison of D13M5 and D13M3 shows a difference, however not statistically significant. Increasing the number of experiments may reveal a significant difference which will be in accordance with the current understanding of preferential strand incorporation into RISC [92,93]. We thus chose the D13M5 hairpin (hereinafter called mirGE) as the best miRNA configuration to compare to the miR-30 hairpin in knock-down experiments targeting CCR5.

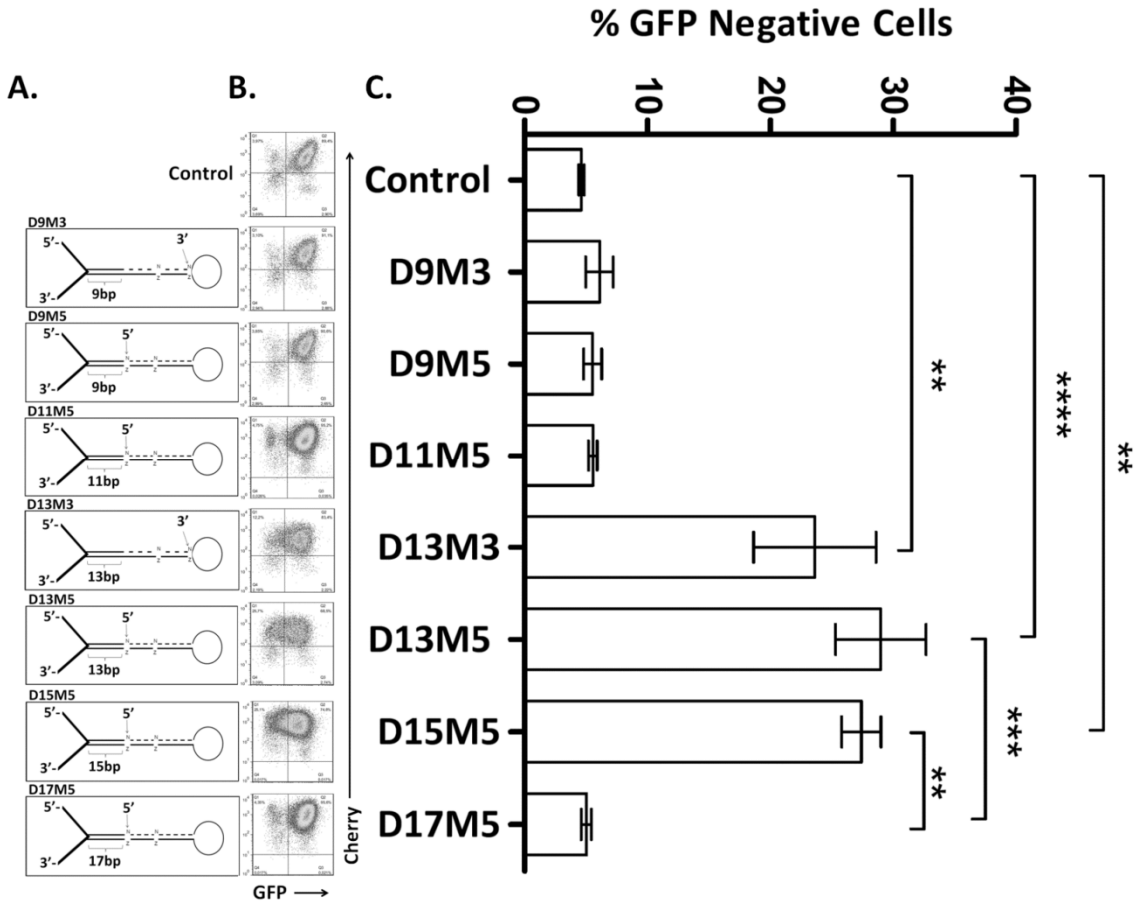


Figure 1. Down regulation of GFP in 4.5 cells using lentivectors expressing various anti-GFP miRNAs constructs. (a) Schematic diagram of variations introduced in the miRNA design, based on features described in Supplementary Figure S2. (b) FACS plots representative of independent experiments: Control, D9M3, D9M5, D11M5 and D13M3 (n=4), D13M5 (n=7). D15M5, D17M5 (n=3). 4.5 GFP expressing cells were transduced with equivalent quantities of lentivectors expressing mCherry alone (GFP control, upper plot), or mCherry together with the corresponding miRNA hairpin displayed alongside in panel A. For analysis, FACS plots were analyzed using quadrants to delineate Cherry-positive transduced cells. Then, the ratio was calculated as the fraction of GFP-negative/Cherry-positive cells over the total of Cherry-positive cells. (c) Bar graph diagram corresponding to the different experiments as illustrated in the FACS plots alongside in panl B, showing the percentage of GFP-negative cells within total Cherry-positive cells for each corresponding miRNA design. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, using 1 way ANOVA followed by Bonferronis Mulitple Comparison Test.

Effect of targeting sequence and hairpin context on CCR5 knock-down.

We then compared the efficiency of mirGE and miR-30 hairpin (for details see **Fig. 7**), context for the knock-down of a second target gene, namely CCR5. Two CCR5 targeting sequences, T1 and T7, were identified *in silico* (see **Supplementary Material, Algorithm**). The T7 target sequence has also previously been described as a very potent target [94]. T1 and T7 were cloned as a single hairpin in either the miR-30 or miGE backbones. As shown in **Supplementary Fig. S3**, the lentivector expression cassette transcribes a single mRNA that encodes for the GFP living color followed by the CCR5 targeting miRNAs. Lentivectors are then used to transduce a clone of HeLa cells constitutively expressing CCR5 (HR5 cells), and CCR5 knock-down is measured in GFP-positive transduced cells (**Fig. 2a**). We found that T1 as well as the T7 targeting sequence were much more efficient in down-regulating CCR5 in the mirGE than in the miR-30 backbone (**Fig. 2b**) and that the T7 was superior to the T1 in the same hairpin context.

Note that the mean fluorescence intensity (MFI) of GFP decreases more in parallel with the knock-down efficiency (**Fig. 2,** in particular in mirGE-transduced cells). This is due to the fact that - in this construct - the hairpins are within the 3'UTR of the mRNAs coding for GFP (see **Supplementary Fig. S3**). Therefore efficient miRNA processing removes poly-A tail leading to de-stabilization of mRNAs coding for GFP [95]. A similar finding was described by Sun et al., [81].

This set of experiments indicates that T7 is a better CCR5 target than T1, and that the mirGE backbone is more efficiently processed by Drosha than the miR-30 backbone.

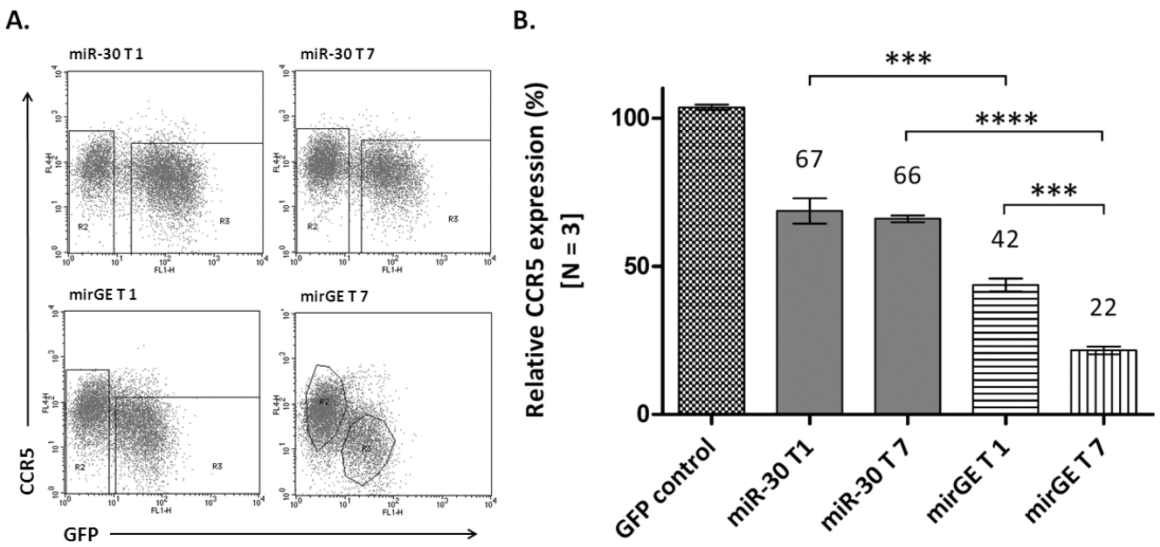


Figure 2. Downregulation of CCR5 in HR5 cells using lentivectors expressing different anti-CCR5 miRNA target sequences in different hairpin contexts. (a) FACS plots showing representative data from three independent experiments performed with three independent lentivector preparations. HR5 cells were transduced with lentivectors expressing either GFP alone (GFP control vector, not shown), or GFP followed by miR-30 or mirGE hairpins containing the T1 or the T7 targeting sequence (see Results and Materials and Methods for details). For determination of MFI (mean of fluorescence intensity) of transduced versus untransduced cells, square gates were used by default and polygonal gates were used when populations were overlapping in one or 2 axes. MFI of GFP for the transduced population (R3) for the miR-30 T1 condition was: 150 (percentage transduction 66%), miR-30 T7 condition: 103.5 (percentage trandsuction 46%), mirGE T1 condition: 47.8 (percentage transduction 48%) and mirGE T7 condition: 30.4 (percentage transduction 23%). (b) Bar graph analysis of data in A. CCR5 downregulation was calculated by dividing the CCR5 MFI value of GFP-positive cells (transduced cells, gate R3) by the CCR5 MFI value of GFP-negative cells (untransduced cells, gate R2), within each sample. This allows for compensation of sample-to-sample variations due to slight changes in CCR5 antibody-to-cell ratios. The relative CCR5 expression in transduced cells was then displayed as percentage of CCR5 expression normalized to the internal control provided by untransduced cells. Values represent average from N=3 independent experiments. ***$p < 0.001$, ****$p < 0.0001$ using 1 way ANOVA followed by Bonferronis Mulitple Comparison Test.

Effect of miRNA backbone and hairpin number on CCR5 knock-down.

We then analyzed the knock-down potential of T7 targeting sequence in HR5 cells when miRNA hairpins are expressed as multiple copies on a single mRNA, either in miR-30 or mirGE context. For this, we used a system that allowed us to clone several copies of miRNA hairpins in tandem in a single lentivector backbone. This strategy, depicted in **Supplementary Fig. S3**, is largely inspired from Sun et al., [81].

Since our ultimate goal is to apply miRNA-based gene silencing for clinical applications, we analyzed experiments performed in conditions which represent the safest transduction rate for gene therapy, i.e. one copy of transgene per cell. According to Poisson's law, when less than 20% of cells are transduced, the majority of transduced cells contain only one copy of the lentivector transgene. So, not only it reproduces the most desirable situation for clinical application but it also facilitates the comparison of different constructs between each other.

We first assessed the efficiency of the miR-30 backbone containing one or two T7 constructs (**Fig. 3**). They gave unsatisfactory results with a knock-down of CCR5 of 16% or 33%, respectively. In contrast, a single hairpin of T7 in the mirGE context was already more efficient than two copies of miR-30 with a knock-down of 45% compared to 33%. Increasing the numbers of hairpins in mirGE to two or three resulted in 80% and 91% knock-down, respectively.

As observed in **Fig. 2**, higher knock-down efficiency of mirGE backbones was correlated with a more important decrease in GFP fluorescence as measured by MFI of GFP, again suggesting that a better Drosha processing results in fewer copies of translatable mRNA. When the mRNA only contains the GFP coding sequence, GFP MFI is 195. When containing GFP and one copy of miR-30 hairpin, the GFP MFI drops to 99 and when containing GFP and one copy of mirGE, GFP MFI drops to 36. From this, one can estimate that only 50% of all mRNA containing one miR-30 hairpin is processed and available for RNA interference, whereas when one copy of mirGE is present, more than 80% of mRNAs are processed and available for RNA interference.

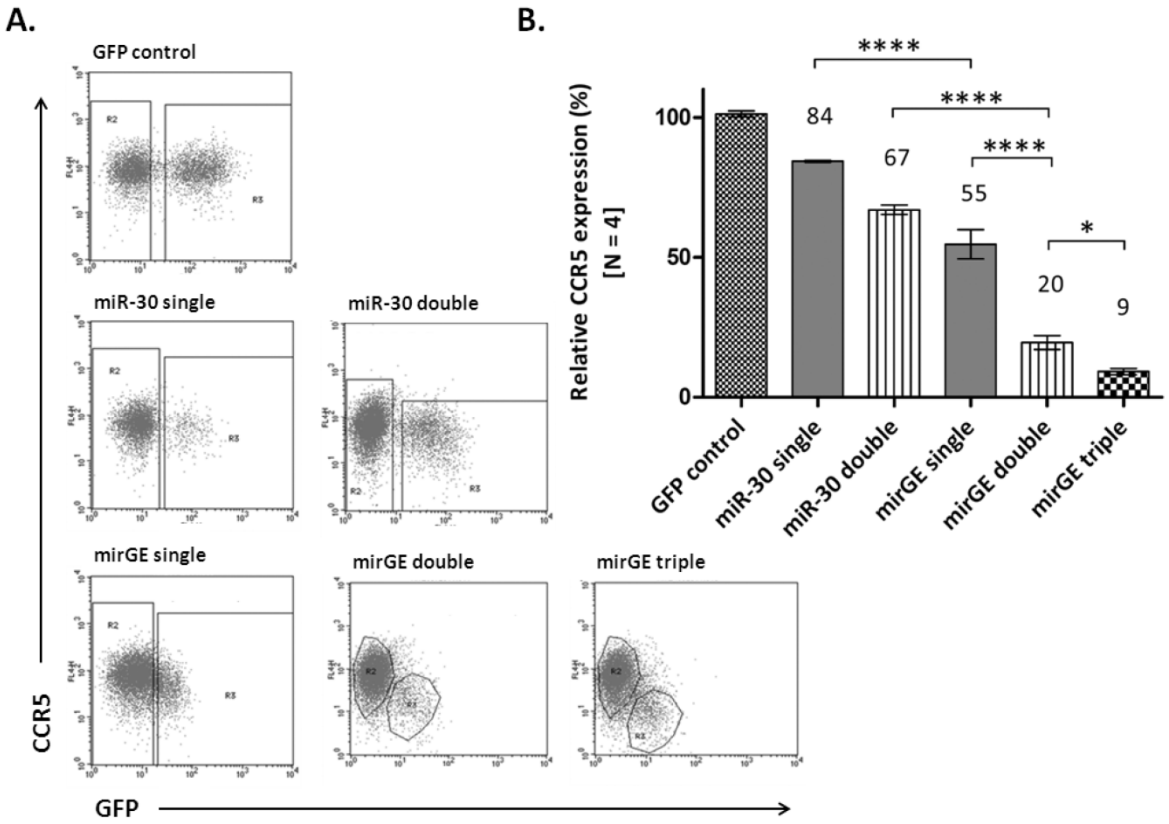


Figure 3. Downregulation of CCR5 in HR5 cells using lentivectors containing various copies of different anti-CCR5 miRNA constructs. (a) FACS plots showing representative data from four independent experiments (n=4). HR5 cells were transduced with various concentrations of lentivectors expressing either GFP alone (GFP control vector), or GFP followed by one or several copies of a given miRNA backbone containing the T7 targeting sequence. The T7 targeting sequence was inserted in one or two copies in the miR-30 context, and in one, 2 or 3 copies in the mirGE context. For further analysis, only FACS plots displaying less than 20% transduced cells (hence mostly single-copy GFP-positive cells) were retained. For determination of MFI (mean of fluorescence intensity) of transduced versus untransduced cells, square gates were used by default and polygonal gates were used when populations were overlapping in one or 2 axes. MFI of GFP for the transduced population (R3) for the GFP control condition was: 195.7 (percentage transduction 38.3%), miR-30 single: 98.8 (percentage transduction 7.6%), miR-30 double: 62.37 (percentage transduction 19.7%), mirGE single: 36 (percentage transduction 13.7%), mirGE double: 18.5 (percentage transduction 7.1%) and mirGE triple: 12.9 (percentage transduction 9.1%). (b) Bar graph analysis of data in A. CCR5 downregulation was calculated as described in Figure 3. The relative CCR5 expression in transduced cells is displayed as percentage of CCR5 expression normalized to internal control untransduced cells. Values represent average from N=4 independent experiments. ****$p < 0.0001$ using 1 way ANOVA followed by Bonferronis Mulitple Comparison Test.

High-throughput sequencing of mirGE and miR-30 small RNA products.

The lower Drosha processing of miR-30 could suffice to account for its lower knock-down efficiency as compared to mirGE. However, another scenario can also contribute to the lower efficiency of miR-30. As discussed for **Fig. 1**, if Drosha processes miR-30 after ~13 base pair of lower stem structure, it will generate a targeting strand that will miss two nucleotides complementary to the target sequence at its 3' end and will also have an extra AU artificial sequence at its 5' end.

To investigate this cleavage issue, we performed a high-throughput sequencing analysis of the small RNAs generated by miR-30 and mirGE constructs. As described in Materials and Methods, we purified fragments in the range of 15 to 40 nt long before sequencing, in order to analyze mostly RNAs that are incorporated into RISC. The raw counts of reads from this sequencing are shown in **Supplementary Table S1** and can also be downloaded as described in Materials and Methods. As shown in **Supplementary Table S2**, the percentage of reads assigned to either miR-30 or mirGE are comparable, i.e. 2.33% vs. 2.21%, respectively. Since mirGE cells are transduced at 23% and miR-30 cells are transduced at 43%, and according to Poisson's law, the average copy number in mirGE cells can be estimated at 0.23 whereas the average copy number in miR-30 cells can be estimated at more than 0.4. This indicates that mirGE can produce approximately 2-times more targeting strands than miR-30. This is in accordance with GFP intensities observed in Figure 3, suggesting that Drosha cleavage is more efficient on mRNAs containing mirGE hairpins than on mRNAs containing miR-30 hairpins.

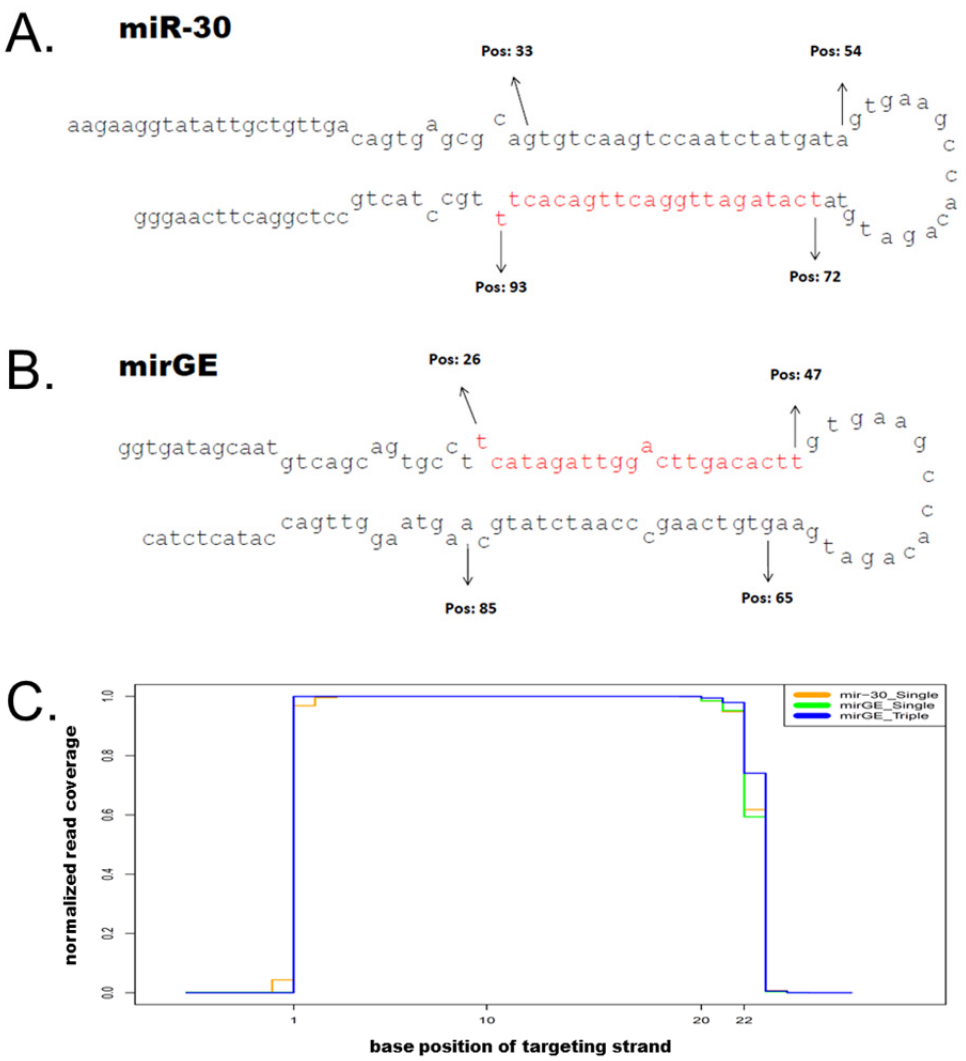
Moreover, the percentage of reads where the targeting strand has an incorrect start position is 7.53 for miR-30 and 0.16 for mirGE, indicating that overall processing of RISC-incorporated targeting strands is approximately 50-times better for mirGE than for miR-30. This is confirmed in the mirGE_triple sample, where the transduction rate is high, 90% positive cells corresponding to an estimated average copy number of more than 5. In this case, even though the reads assigned to mirGE represent more than two thirds of all cellular reads (69.8%), the

percentage of reads with an incorrect start position is even lower than in mirGE_single (0.06% vs. 0.16%, respectively). This suggests that the mirGE backbone can be processed with higher efficiency and higher precision than miR-30, even when representing the majority of all Drosha-processed miRNAs. We further investigated the issue of cleavage accuracy for the mirGE or miR-30 backbone. As shown in **Fig. 4**, the Drosha cleavage occurs at the correct position both in mirGE and miR-30 although the accuracy with which this occurs is very different when comparing the two backbones (discussed below). This is surprising for miR-30 since its lower stem is two nucleotides shorter than usually seen in miRNAs [72]. Also, Dicer cutting occurs mostly at the expected site so the vast majority of the reads covers the targeting strand, whether it is generated by mirGE or by miR-30.

However, this analysis identified two further points where mirGE was superior to miR-30. First, there is a 10-fold difference in the ratio of RISC incorporation of targeting vs. passenger in favor of mirGE over miR-30. As shown in **Supplementary Table S1** and **Fig. 4**, for mirGE_single sample, there is a count of 18253 reads at position 26 vs. no read at position 65 or around. Thus, a ratio of 14'823 could only be calculated with the mirGE_triple sample (2'638'464 reads starting at position 26 over 178 reads starting at position 65). For miR-30, there are 44'853 reads of starting at position 72 over 33 reads starting at position 33, giving a ratio of targeting strand/passenger strand of 1359. Second, Drosha cutting is 500 to 1'000 fold more precise in mirGE than in miR-30. As shown in **Supplementary Table S1** and **Fig. 4**, for mirGE_single sample, there is a count of 18253 reads at position 26 and a total of 5 reads proximal but outside position 26, giving a ratio of 0.00027 of incorrect cuts. A similar ratio was obtained from the mirGE_triple sample, with 2'638'464 reads at position 26 and a total of 1194 reads proximal but outside position 26, giving a ratio of 0.00045. Of note, even when reads are coming from mirGE processing of a triple hairpin and amount to more than two thirds of all trimmed reads, Drosha still manage to be extremely accurate in mirGE processing. On the contrary, miR-30 processing by Drosha is 500 to 1'000 times less precise with a ratio of 0.21 of incorrect cuts (33 reads at position 33 and 7 reads at position 34). When applying this calculation to Dicer

processing, miR-30 appears to be processed with an accuracy that is 6-times higher than for mirGE, with ratios of incorrect cuts of 0.08 and 0.49, respectively. Such a difference is hard to explain since both hairpins have the same loop to present to Dicer.

Taken together, this high-throughput sequencing analysis shows that mirGE is superior to miR-30 in four aspects: yield of targeting strand incorporation into RISC; percentage of RISC-incorporated targeting strands with the correct sequence; precision of cleavage by Drosha and ratio of targeting strand over passenger strand. It thus provides molecular basis to account for the superiority of mirGE over miR-30 in knock-down efficiency.



D.

	Ratio of Drosha incorrect cuts (position 26/33)	Ratio of Dicer incorrect cuts (position 65/72)	Ratio of targeting strand/passenger strand
miR-30_single	0.21	0.08	1'359
mirGE_single	0.00027	N/A	N/A
mirGE_triple	0.00045	0.49	14'823

Figure 4. High-throughput sequencing of short RNAs generated by miR-30 and mirGE and miRNA constructs. (a) Schematic diagram of the miR-30 miRNA containing the CCR5 T7 target. Targeting strand is in red (bottom strand). Position and numbering of reads major starts and reads major ends of coverage (see Supplementary Table S1 for details) are indicated by arrows. (b) Schematic diagram of the mirGE miRNA containing the CCR5 T7 target. Targeting strand is in red (top strand). Position and numbering of reads major starts and reads major ends of coverage (see Supplementary Table S1 for details) are indicated by arrows. (c) Coverage plot of reads aligning to the targeting strands of miR-30 or mirGE hairpins sequences. Base 1 corresponds to nucleotide 72 for miR-30 and to nucleotide 26 for mirGE. (d) Summary of analysis of reads covering the targeting or passenger strand sequences (see text and Supplementary Table S1 for details).

Effect of CCR5 knock-down on HIV infection *in vitro.*

Finally, we wanted to determine whether the CCR5 knock-down induced by transduction of cells with a single copy of lentivector containing an optimized anti-CCR5 miRNA design was efficient enough to render cells resistant to HIV infection. For this, we used a HIV-permissive clone of HeLa cells expressing both CD4 and CCR5 (HR5 cells, see Materials and Methods section). We transduced the HR5 cells with varying amounts of lentivectors, containing GFP and triple hairpin anti-CCR5 mirGE, or GFP only as negative control (**Fig. 5a**). Again, we included a condition with ≤20% of the cells transduced (mirGE 2 µl = 13% and 1 µl = 6%), hence observing the effect of single-copy transgene expression. Cells were seeded at 20% confluence one day prior to infection with the CCR5 tropic HIV strain, YU-2. All cells which were either mock-transduced or transduced with the GFP control lentiviral vector were either dead or showed massive syncytia induction 5 days post infection (**Fig. 5a**). In contrast, a sub-population of the cells previously transduced with the anti-CCR5 mirGE lentivector survived the HIV infection and further proliferated. Monitoring of the HIV p24 antigen in the supernatants reflected the microscopic findings with lower levels of p24 antigen in the cells transduced with the anti-CCR5 mirGE (**Fig. 5b**). At day 5, we observed syncytia induction in close proximity to apparently healthy cells. At day 7, the positive selection for the HIV resistant cells is evident. Phenotypic analysis of these surviving cells shows that they have lost CCR5 expression (**Fig. 5g**). The cells which survived this first infection with R5-tropic HIV were re-challenged with the same HIV strain. Corroborating our observation that these cells have lost CCR5 expression, they were entirely HIV-resistant (**Fig. 5c**). Finally, we wanted to verify that the 2 rounds of R5-tropic HIV infection did not select for cells that were resistant to HIV infection in general. For this, we infected the surviving cells with an X4-tropic HIV strain. As shown in **Fig. 5d**, these cells can support X4-tropic HIV replication at the same level as naïve HR5 cells. Thus, no non-specific HIV resistance appears to have developed in the process. We also performed a phenotypic analysis of cells that survived R5-tropic infection and compared them to the sub-population of cells transduced with the anti-CCR5 lentivector before infection. As shown in **Fig. 5f**, the transduced

cells represent 6% of the total population, which implies that the vast majority contain only one copy of the transgene. When compared to the internal negative control provided by the untransduced cells whose CCR5 MFI is 114, the knock-down ratio is more than 10 fold, as also shown in **Fig. 3b**. The same phenotypic analysis performed seven days after HIV R5 infection shows a dramatic enrichment, yielding to a population where CCR5 expression is virtually absent (**Fig. 5g**).

To further check if our anti-CCR5 lentivector could inhibit HIV infection in natural target of the virus, we performed HIV-R5 infection in human monocyte-derived macrophages (MDMs) with or without prior transduction with the anti-CCR5 T7 triple hairpin lentivector described above. MDMs were matured in culture for 8 days as described in the Materials and Methods section. In order to maximize R5-tropic HIV infection, MDMs were treated with VPX-virus like particles for two hours prior to transduction, as described previously [96]. The MDMs were then transduced with various quantities of either a control GFP vector or the anti-CCR5 lentivector, followed four days after by infection with R5-tropic HIV-Luciferase virions (see Materials and Methods for details). As shown in **Fig. 6**, we can see a strong inhibition of HIV infection using anti-CCR5 lentivector at a MOI of 1.7. Other conditions using lentivectors, i.e. lower MOI of anti-CCR5 lentivector or control GFP lentivector display a moderate inhibition of HIV infection, as compared to the *no vector* condition. This is most likely due to the triggering by defective particle of cell-autonomous innate immune systems independent of SAMHD1 (see [97] for review).

Altogether, these data show that our anti-CCR5 lentivector can efficiently block HIV infection at low copy number in both artificial and natural target cells.

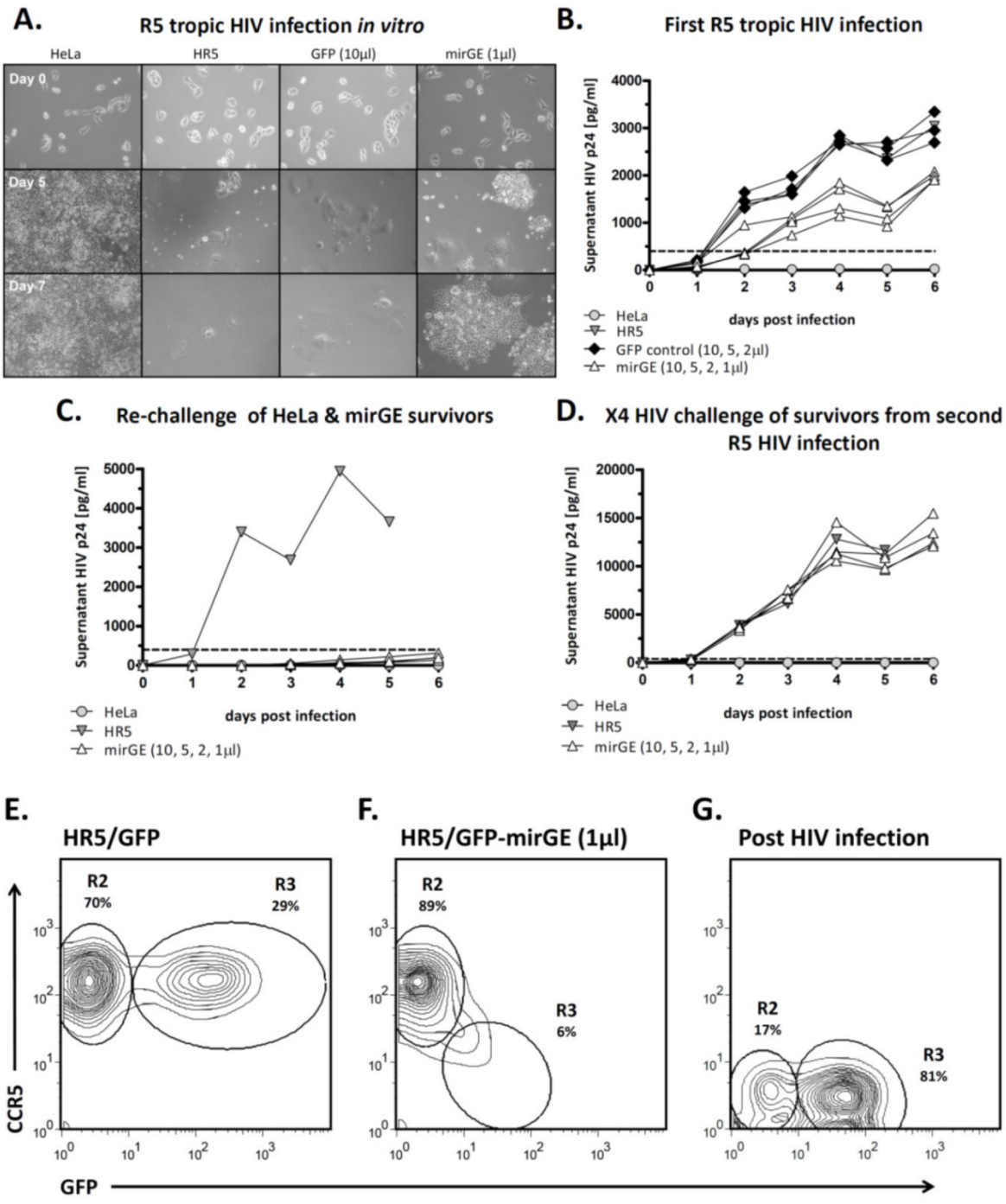


Figure 5. Resistance to R5-tropic HIV infection of HR5 cells transduced with anti-CCR5 mirGE lentivector. (a) Representative images of control parental HeLa cells (HeLa), untransduced HR5 (HR5), HR5 cells transduced with a lentivector expressing GFP alone (GFP), and HR5 cells transduced with a lentivector expressing GFP and 3 hairpins of anti-CCR5 mirGE (mirGE). Pictures of these four cell lines were taken at day 0, day 5 and day 7 of HIV-R5 infection. Only parental HeLa cells and mirGE-transduced cells survive the infection. Note that mirGE 1µl indicates cells transduced with the lowest volume of vector corresponding to a 7% transduction rate. (b) Values of HIV p24 antigen in supernatants of cells showed in Figure 5a, collected over 6 days post R5 HIV infection. (c). Values of HIV p24 antigen collected over 6 days

post R5 HIV infection, in supernatants of mirGE cells that survived the first HIV challenge in Figure 5b. Negative and positive controls for HIV replication were provided by naïve HeLa and HR5 cells, respectively. (d) Values of HIV p24 antigen collected over 6 days post X4 HIV infection, in supernatants of mirGE cells that survived the first and second HIV challenges in Figure 5b and c. Negative control for X4 HIV infection was provided by naïve CD4-negative parental HeLa cells and a fresh batch of naïve HR5 cells served as the positive control for infection. (e) Phenotypic analysis of HR5 cells transduced with GFP control lentivector (no miRNA). (f) Phenotypic analysis of HR5 cells transduced with GFP-anti-CCR5 mirGE lentivector prior to R5-tropic HIV infection. Within the untransduced cells in gate R2 (89%), the CCR5 and GFP MFIs are 144 and 2.4, respectively. Within the transduced cells in gate R3 (6%), the CCR5 and GFP MFIs are 14 and 18, respectively. (g) Phenotypic analysis of HR5 cells from (F) 7 days post infection with R5-tropic HIV. The majority of cells (81%) are CCR5-negative. The CCR5 and GFP MFIs in this population (gate R3) are 3 and 48, respectively.

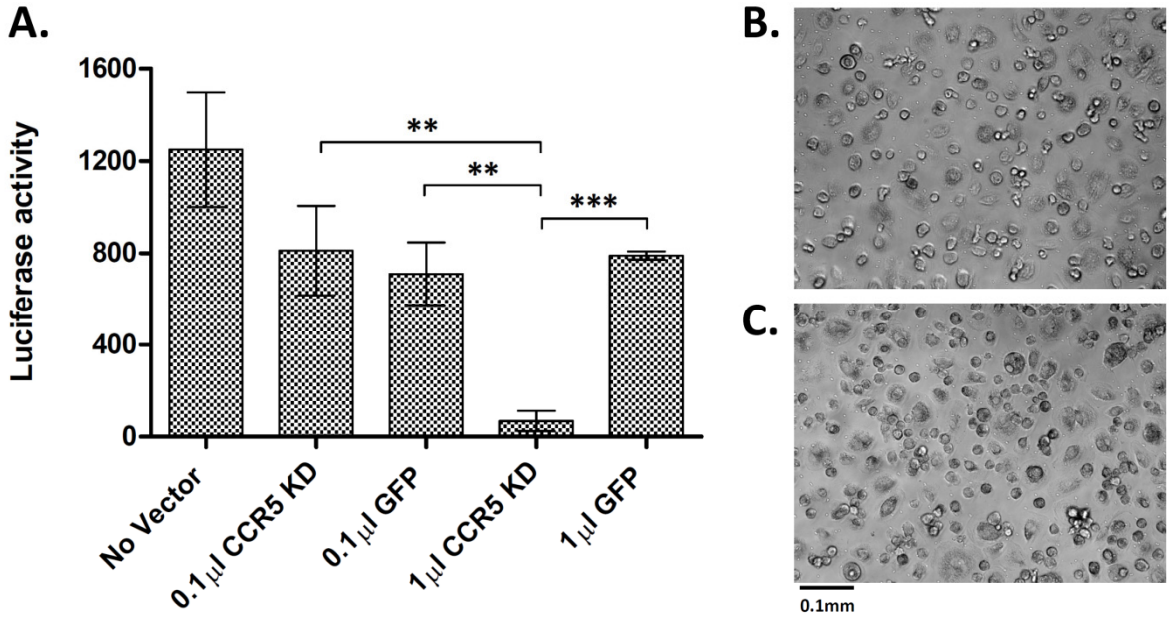


Figure 6. Resistance to R5-tropic HIV infection of Monocyte-derived Macrophages (MDMs) transduced with anti-CCR5 mirGE lentivector. (a) Bar graphs showing the level of infection of MDMs by recombinant R5-tropic HIV particles encoding luciferase (see Materials and Methods for details). Prior to infection, MDMs were either not transduced (no vector) or transduced with various amounts of anti-CCR5 mirGE lentivector (CCR5 KD) or with a control vector expressing only GFP (GFP). Vector stocks (CCR5 KD and GFP) were normalized and 1 µl of vector corresponds to a MOI of 1.7. Experiments consisted of a minimum of 3 technical replicates. Luciferase activity: No vector 1250 (sem ± 248), 0.1ul CCR5 KD vector 811 (sem ± 195), 0.1ul GFP vector 711 (sem ± 137), 1ul CCR5 KD vector 69 (sem ± 44) and 1ul GFP vector 791 (sem ± 18). P values (**p<0.0099, **p<0.0038, *** p<0.0004) were obtained using a two-tailed unpaired *t*-test. (b-c) Light microscope images of macrophages 4 days post transduction with 1µl of anti-CCR mirGE vector or 1µl of GFP vector, respectively (20x magnification).

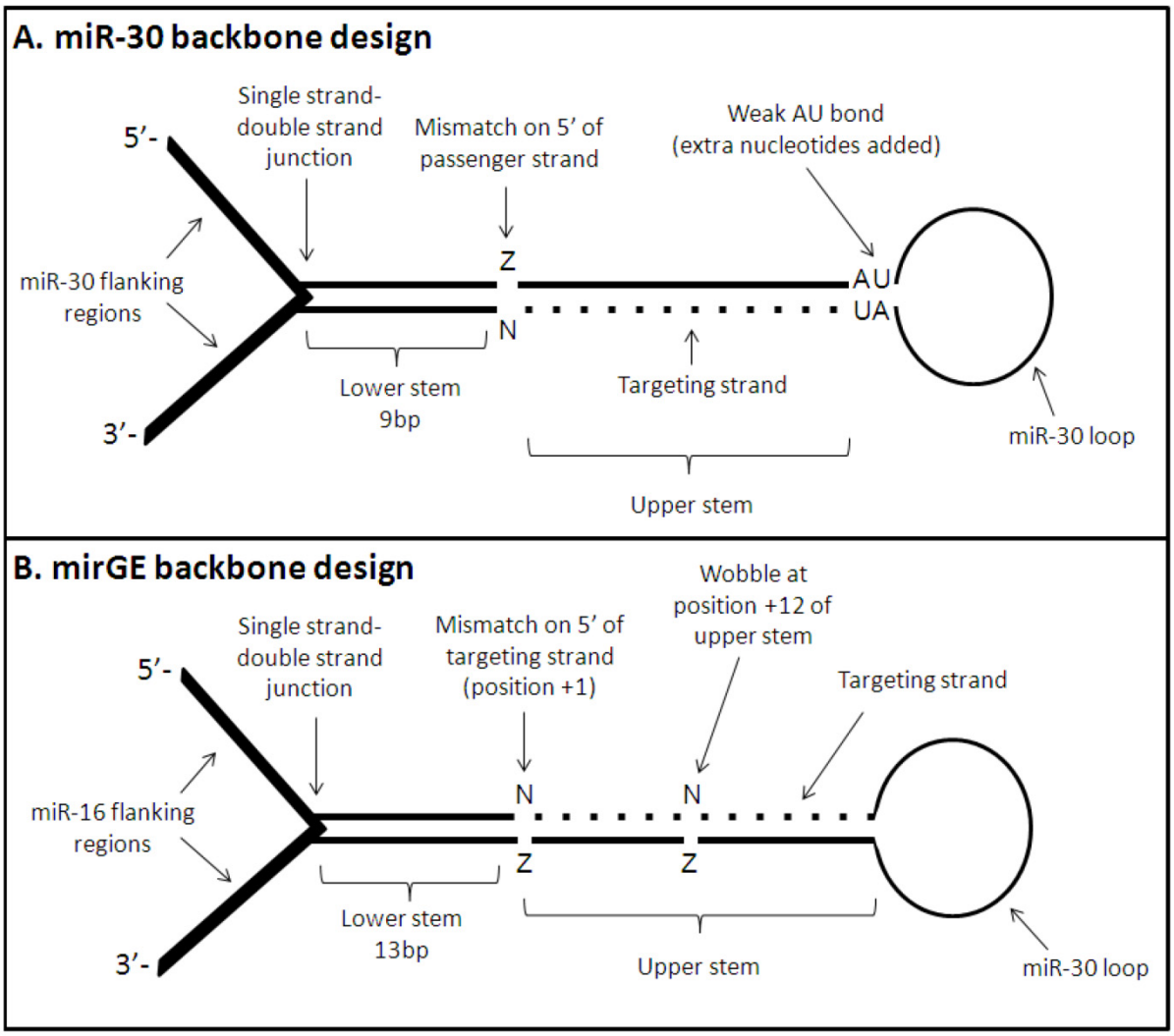


Figure 7. Schematic representations of miR-30 and mirGE designs. (a) miR-30 design as it has been previously described [1] [17] [14]. Main features include 11 bp lower stem, upper double-strand stem composed of the fully complementary targeting strand (bottom strand, dotted line), 3' mismatch of targeting strand as well as a weak AU bond on the 5' of the targeting strand. (b) mirGE backbone developed in this study. Main features include miR-16 flanking regions, upper double-strand stem composed of the fully complementary targeting strand (top strand, dotted line), a 13bp lower stem , a wobble at position 12 of the upper stem (position 1 is the first 5' base of the targeting strand), a 5' mismatch and no 3' mismatch on the targeting strand.

DISCUSSION

The work presented in this paper is the result of an ambitious goal: Efficient gene knock-down with a single copy of miRNA-containing lentivector. We could achieve this goal by optimization of critical miRNA features, combined with multimerization of miRNA hairpins in a single cassette. When applied to human CCR5, we reached a knock-down efficiency which provides the ground for a clinically-applicable approach towards cellular immunization against HIV.

When we designed our knock-down lentivector constructs by implementing the miR-30 protocols from institutional on-line tools (http://hannonlab.cshl.edu), these constructs hardly reduced the level of CCR5 expression by one third (**Fig. 2**, miR-30 T1 and miR-30 T7). This was surprising and disappointing since T7 has been described as one of the most potent CCR5 RNAi targets [94]. Notably, cells with multiple copies of anti-CCR5 T7 miR-30 did show efficient CCR5 knock-down. Clearly, one cannot expect any phenotype with this level of knock-down, and therefore no clinical applicability.

A careful review of articles about Drosha processing and RISC incorporation identified three potential features critical for optimal miRNA-based silencing: (1) length of the lower stem for optimal Drosha processing, (2) destabilization of the 5' end of the targeting strand for optimal RISC incorporation, and (3) a mismatch in the middle of the upper stem to avoid abortive processing by Drosha [67,72,77,81,82,98-100].

When these three features were incorporated in our mirGE hairpin design, the knockdown efficiency improved dramatically compared to miR-30-based hairpin designs, whether we targeted GFP or CCR5 (**Fig. 1** and **2**). The level of knock-down with a single mirGE hairpin construct, however still did not reach a level of down-regulation compatible with clinical applications i.e. > 90%. We reached this threshold by tandem addition of hairpins, achieving 90% knock-down with a single copy of a lentivector containing a triple anti-CCR5 mirGE construct (**Fig. 3**). These tandem repeats do not seem to be subject to recombination (data not

shown) and are thus a safe alternative to the accumulation of integrated proviral copies in the target cell genome.

To investigate the mechanisms underlying the superiority of mirGE over miR-30, we performed a high-throughput sequencing of short RNAs generated by the two miRNA backbones (**Fig. 4**). We found that mirGE performed better than miR-30 in terms of yield of targeting strand incorporation into RISC as well as percentage of correct targeting strands incorporated into RISC. Also, the ratio of targeting strand over passenger strand is higher for mirGE than for miR-30 . We also found that Drosha cutting on miR-30 was quite imprecise, with 21% of cuts outside of the correct position. On the contrary, Drosha cutting on mirGE was very precise (500 to 1'000-fold more precise than miR-30) and keeps the same level of accuracy even when mirGE is expressed at high levels and in a triple hairpin context.

Surprisingly, Dicer cutting was not very precise, ranging from 8% to 49% of incorrect cuts for miR-30 and mirGE, respectively. This level of inaccuracy is comparable to Drosha cutting on miR-30 (21%). One possibility is that the miR-30 loop that we kept in our mirGE design is also not optimal. We plan on testing other loop sequences based on features affecting enzymatic processing of hairpin transcripts as described elsewhere [101].

Taken together, our results show a clear superiority of mirGE over miR-30 in terms of processing, cleavage accuracy, targeting strand incorporation and, most of all, knockdown efficiency. Also, in mirGE, the upper strand is incorporated in RISC, whereas in miR-30 it is the lower strand. In mirGE, Drosha cutting generates the 5' end of the guide strand and is extremely precise, whereas in miR-30, the terminal loop processing, whether done by Dicer or single-strand RNAses, generates the 5' end of the guide strand and is imprecise and requires further optimization. Finally, in the meantine, we have applied the mirGE design to other target genes (Caveolin-1, VEGF and Wnt5a, data not shown) and found equally efficient knock-down efficiency, allowing gene repression at low copy number. We thus propose to use the mirGE design as default design for miRNA-based knock-down applications.

Using a triple anti-CCR5 mirGE construct to protect Hela R5 cells against HIV infection *in vitro,* we showed that a sub-population of cells transduced at levels lower than 10%, hence mostly containing only one copy of anti-CCR5 mirGE [89], could resist HIV R5 infection (**Fig. 5**). Re-challenging these surviving cells with HIV R5 did not show any virus replication, whereas the same cells remained permissive to HIV X4 infection, proving the specificity of the anti-CCR5 cellular immunization. To further validate the protective effect of our anti-CCR5 constructs, we tested it in natural targets of HIV R5, i.e. human macrophages (**Fig. 6**). We found that our construct, even at low MOI, efficiently and specifically protected macrophages against HIV-R5 infection. This protective effect in primary human cells was later confirmed in humanized mice (Myburgh et al., 2014 manuscript in preparation).

Altogether, these results can be compared in terms of efficiency to the work of Ringpis et al., [55]. However, they used an shRNA-based vector which may be less desirable for clinical application due to potential adverse effect of shRNA-related toxicity [63-65].

In conclusion, this work provides the ground to ultimately build a genetic tool that can be used to confer HIV-resistance in patients. Also, the efficiency of our system provides a universal tool to knock-down any clinically-relevant target gene without sacrificing safety by requiring multiple transgene integrations. Finally, we provide here an algorithm (see **Supplementary Material: Algorithm for mirGE design**) as well as a set of genetic tools for everyone to design "optimized" miRNA against any target gene.

MATERIALS AND METHODS

Construction of miRNA-containing plasmids and lentiviral vectors: To construct the plasmid intermediates containing the various miRNA hairpins, we followed a protocol largely inspired by Sun et al. with the following modifications [81]. An amplicon containing the miRNA hairpin flanked by sequences for digestion with restriction enzymes was generated by high fidelity PCR using Herculase II polymerase (Agilent). The oligos for PCR template and primers were obtained from Microsynth then Sigma. The templates for miR-30 hairpins had inside sequences specific for gene targeting and common outside sequences for annealing with miR-30 primers (see Supplementary Material). The mirGE hairpins were amplified from similar templates but with common outside sequences for annealing with mirGE primers (see Supplementary Material). PCR products were digested with BamHI and XbaI restriction enzymes (New England Biolabs), and ligated into a pENTR-derived plasmid (Invitrogen) using BamHI and XbaI sites located directly, either downstream of the MGST2 gene (Genbank Access U77604.1) for miRNAs targeting GFP (see Supplementary Figure S1a), or downstream of the GFP gene for miRNAs targeting hCCR5 (Supplementary Figure S3a). Ligation was performed using T4 DNA ligase (New England Biolabs). The pENTR-GFPmir plasmid maps and sequences are available at our institutional website (http://medweb2.unige.ch/salmon/lentilab). The amplicon parts of each clone were verified by sequencing. For constructs containing multiple hairpins, PCR products were inserted using BamHI and SpeI sites as depicted in Supplementary Figure S3a and described before by Sun et al. (CCR5 knockdown) [81]. This allows for the insertion of multiple miRNAs where each new addition was verified by sequencing. The final lentivector plasmid was generated by an LR Clonase II (Invitrogen) mediated recombination of a pENTR plasmid containing the human ubiquitin promoter (pENTR-L4-UBI-L1R) and a pCLX-R4-DEST-R2 lentivector destination cassette (Supplementary Figure S1b and S3b). For GFP knock-down experiments, a specific lentivector destination cassette (pCWX-R4-DEST-R2-PC) was used, containing an additional transcription unit with the human PGK promoter driving the mCherry living color, as described in the text. All maps and sequences of the plasmids used in this study

are available at our institutional website (http://medweb2.unige.ch/salmon/lentilab). The GFP target sequence –AAGAACGGCATCAAGGTGAACT- was taken from previous publication [102]. The human CCR5 (Genbank NM_000579.3) target sequences were chosen using the online webserver at http://rna.tbi.univie.ac.at/cgi-bin/RNAxs, and from published article [94]. The two CCR5 target sequences retained were named Target 1 (T1) 5'-TTTCCATACAGTCAGTATCAAT and Target 7 (T7) 5'-AAGTGTCAAGTCCAATCTATGA. Sequences of templates for various anti-GFP miRNA and anti- CCR5-T1, anti-CCR5-T7, used for PCR amplification, as well as the full algorithm used to design optimized upper and lower strands, are given in Supplementary Material section.

Lentiviral vector production and titration: Lentiviral vector stocks were generated using transient transfection of HEK 293T/17 cells with the specific lentivector transfer plasmid, the psPAX2 plasmid encoding gag/pol and the pCAG-VSVG envelope plasmid, as previously described [103,104]. Lentivector titer was performed using transduction of HT-1080 cells followed by flow cytometry quantification of GFP-positive (or mCherry positive) cells 5 days after infection, as previously described [103,104].

Cell culture and knock-down analysis: All cell lines were cultured in High-Glucose Dulbecco's modified eagle medium (DMEM, Sigma) supplemented with 10% FCS, 1% Penicillin, 1% Streptomycin and 1% L-Glutamine. For GFP knock-down studies, the 4.5 cell line [90], containing one copy of a GFP-expressing lentivector was used. For each knock-down assay, cells were analyzed 5 days after transduction. For CCR5 knock-down studies, a subclone of Hela-derived TZM-bl cells (AIDS Repository), expressing CD4 and high levels of human CCR5, named here HR5, was used. CCR5 and CD4 quantification was performed using an APC and PE Cy7-labelled antibody, respectively (BD Pharmingen 550856, Biolegend 300511), followed by flow cytometry analysis using FACS Vantage (Becton Dickinson) or MoFlow Astrios (Beckman Coulter).

High-throughput sequencing of miRNA cleavage products: The samples were processed following the illumina TruSeq Small RNA protocol (www.illumina.com). In short, total RNA (2 µg per sample) from R5 cells transduced with lentivectors expressing either mirGE-T7 or miR-30 T7 miRNAs was extracted using TRIzol (Life Technologies). After validation of RNAs, 1µg of total RNA from each sample was ligated to specific 3' and 5' adapters. In a subsequent reverse transcription step, single stranded cDNA was generated, followed by PCR amplification and gel purification that selecting constructs holding specific RNA fragments with an approximate length of 22nt (range 15-40). Reads were sequenced on the illumina HiSeq platform with a length of 50bp. Partial adapter sequences were trimmed using Flexbar. The trimmed reads were aligned to the hairpin sequences of miRBase v20 and the sequences of the mirGE and miR-30 constructs. Alignment was done with Bowtie with the options "--chunkmbs 1024 -e 50 -a -m 50 --best --strata --nomaqround –y". We counted only the number of reads that aligned to the positive strand of the sequences. Raw data of this study have been uploaded to the European Nucleotide Archive and can be accessed at this link; http://www.ebi.ac.uk/ena/data/view/PRJEB7175

R5-tropic and X4-tropic HIV infection of HR5 cells: Viral stocks were obtained by Polyethylenimine (PEI) mediated transfection (Polysciences) of 293T cells with pYU-2 or pNL4-3 (provided through the NIH AIDS Research and Reference Reagent Program). Forty-eight hours after transfection, virus was harvested, filtered (0.45µm), and frozen at -80°C until use. Virus titers were determined as previously described [105]. Briefly, TCID50 (tissue culture infectious dose 50%) was determined by infecting human $CD8^+$ T cell depleted PBMC from three donors which were stimulated by PHA and anti-CD3 beads (Dynal 11131D, Life Technologies). Then, viral stocks were adjusted to 1x106 TCID50/ml, aliquoted and frozen at -80°C before use. HR5 cells were infected for 6 hours at an MOI of 2.5, washed 3 times with PBS and then cultured in DMEM (Invitrogen) with 10% FCS (PAA laboratories), 1% Penicillin/Streptomycin (Invitrogen Life Technologies) and 1% L-glutamine (Invitrogen Life Technologies). Assays for HIV p24

antigen levels in cell culture supernatants were performed using an in-house ELISA as described [106].

R5-tropic HIV-Luciferase infection of Monocytes-derived Macrophages: VPX-encoding virus-like particles (VPX-VLPs) were produced essentially as described previously [107]. Briefly, 293T cells were transfected using polyethylenimin (PEI) (Sigma-Aldrich) with the Vpx-containing pSIV3+ plasmid (provided by A. Cimarelli, Lyon) and the pMD2.G (VSV-G envelope) plasmid. The medium was changed after 16 hours, and supernatants were collected after 48 hours and filtered through 0.45um low protein binding syringe filters. Monocyte-derived Macrophages (MDMs) were prepared as follows. Monocytes from HIV seronegative donors were isolated from total peripheral blood after ACK lysis (Lonza) by CD14 micro-beads (Miltenyi Biotec). The isolated monocytes were matured to produce macrophages for 6 days with X-vivo-10 medium (Lonza) supplemented with 1000U/ml GMCSF and 100ng/ml MCSF. After 6 days, the medium was changed to X-vivo-10 w/o Cytokines for 2 days. VPX-VPLs were added onto the cells for 2 hours, cells were washed and then transduced overnight with the anti-CCR5 mirGE or control GFP lentivectors. The next day the cells were washed and kept in culture for 4 days prior to infection with R5 tropic NL4-3/luciferase virus pseudotyped with ADA envelope virus for 6 hours. After 6 hours cells were washed and new medium added. Cells were lysed 24 hours after infection with the Cell Culture lysis reagent (Luciferase assay System Promega) and luciferase was measure with the MLX microplate luminometer (Dynex technologies).

Statistical Analysis: The statistical analyses were performed using GraphPad Prism 5.04 (GraphPad Software). We used One Way ANOVA followed by Bonferronis Multiple Comparison Test as well as t-test (Non parametric, Mann-Whitney U). In Supplementary Table 2, significant difference of incorrect targeting strand processing was determined using a proportion test (R prop.test; 2-sample test for equality of proportions with continuity correction).For all tests, p 0.05 was taken as statistically significant.

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SUPPLEMENTARY MATERIAL

Primer sequences: The sequences annealing to miR-30 or mirGE templates templates are underlined. The sequences for restriction sites BamHI, SpeI and XbaI are in bold lower case.

miR-30 5' primer: 5'-CAGAAGG**ggatcc**ATCGAT**actagt**GATCCAAGAAGGTATATTGCTGTTGACAGTGAGCG

miR-30 3' primer: 5'-CTAAAGTAGCCCCTT**tctaga**ATCGTAGCCCTTGAAGTCCGAGGCAGTAGGCA

mirGE 5' primer: 5'-CAGAAGG**ggatcc**ATCGAT**actagt**GGTGATAGCAATGTCAGCAGT

mirGE 3' primer: 5'-AGTAGCT**tctaga**GTAGAGTATGGTCAACCTT

Template Sequences: Sequences are shown as they were received, ready to serve as template to PCR amplify with the corresponding primers shown above. Targeting strands are in blue, passenger strands are in red.

miR-30 T1 template:

5'-
GATCCAAGAAGGTATATTGCTGTTGACAGTGAGCGCTTCCATACAGTCAGTATCAATTAGTG
AAGCCACAGATGTAATTGATACTGACTGTATGGAAATGCCTACTGCCTCGGACTTCAAGGGC
TACGAT

miR-30 T7 template:

5'-
GATCCAAGAAGGTATATTGCTGTTGACAGTGAGCGCAGTGTCAAGTCCAATCTATGATAGTG
AAGCCACAGATGTATCATAGATTGGACTTGACACTTTGCCTACTGCCTCGGACTTCAAGGGC
TACGAT

mirGE T1 template:

5'-
GGTGATAGCAATGTCAGCAGTGCCTATTGATACTGACTGTATGGAAAGTGAAGCCACAGATG
TTTCCATACAATCAGTATCAACAAGTAAGGTTGACCATACTCTAC

mirGE T7 template:

5'-
GGTGATAGCAATGTCAGCAGTGCCTTCATAGATTGGACTTGACACTTGTGAAGCCACAGATG
AAGTGTCAAGCCCAATCTATGCAAGTAAGGTTGACCATACTCTAC

D9M3 GFP template:

5’-
GGTGATAGCAATGTCAGCAGTAGTTCACCTTGATGCCGTTCTTGTGAAGCCACAGATGCAGA
ACGGCACCAAGGTGAACTAAGGTTGACCATACTCTAC

D9M5 GFP template:

5’-
GGTGATAGCAATGTCAGCAGTAGTTCACCTTGATGCCGTTCTTGTGAAGCCACAGATGAAGA
ACGGCACCAAGGTGAACCAAGGTTGACCATACTCTAC

D11M5 GFP template:

5’-
GGTGATAGCAATCAGCAGTGCCTAGTTCACCTTGATGCCGTTCTTGTGAAGCCACAGATGAA
GAACGGCACCAAGGTGAACCAAGTAAGGTTGCATACTCTAC

D13M3 GFP template:

5’-
GGTGATAGCAATGTCAGCAGTGCCTAGTTCACCTTGATGCCGTTCTTGTGAAGCCACAGATG
CAGAACGGCACCAAGGTGAACTAAGTAAGGTTGACCATACTCTAC

D13M5 GFP template:

5’-
GGTGATAGCAATGTCAGCAGTGCCTAGTTCACCTTGATGCCGTTCTTGTGAAGCCACAGATG
AAGAACGGCACCAAGGTGAACCAAGTAAGGTTGACCATACTCTAC

D15M5 GFP template:

5’-
GGTGATAGCAATCAGTCAGCAGTGCCTAGTTCACCTTGATGCCGTTCTTGTGAAGCCACAGA
TGAAGAACGGCACCAAGGTGAACCAAGTAAGGTTGACTGCATACTCTAC

D17M5 GFP template:

5’-
GGTGATAGCAATCAGTGTCAGCAGTGCCTAGTTCACCTTGATGCCGTTCTTGTGAAGCCACA
GATGAAGAACGGCACCAAGGTGAACCAAGTAAGGTTGACACTGCATACTCTAC

Algorithm for the design of mirGE-based amplicons for cloning: This algorithm is a compilation of several published and on-line references including [108,109].

1. Copy your target sequence (ORF, 3'UTR, etc).
2. Use the three following web tools to select at least ten target sequences (http://www5.appliedbiosystems.com/tools/siDesign/; http://www.med.nagoya-u.ac.jp/neurogenetics/i_Score/i_score.html; http://rna.tbi.univie.ac.at/cgi-bin/RNAxs
3. Select candidates that are in overlapping pools.
4. Blast each 19mer to check that they are targeting no other gene other than your target gene.
5. Exclude "bad" candidates by following instructions of [108], page 12.
6. Extend 19mer to 22mer by adding nucleotides from the original target sequence, either upstream or downstream, favoring a resulting complementary sequence that will have a T on the 5' end [109] . This 22mer is the basis for the following shRNA design.
7. Make a complementary strand to this 22mer.
8. To favor incorporation of the targeting strand into RISC, it needs to have one flapping nucleotide at its 5' end. For human CCR5 mRNA (GenBank: NM_000579.3), the result is TTTCCATACAGTCAGTATCAAT (22mer).
9. Make an antiparallel strand, which will be the top strand of mirGE context, i.e the targeting strand. To be efficiently incorporated into RISC, the 5' terminus A needs to flap, thus make a wobble on the complementary strand (Z)

5'-ATTGATACTGACTGTATGGAAA

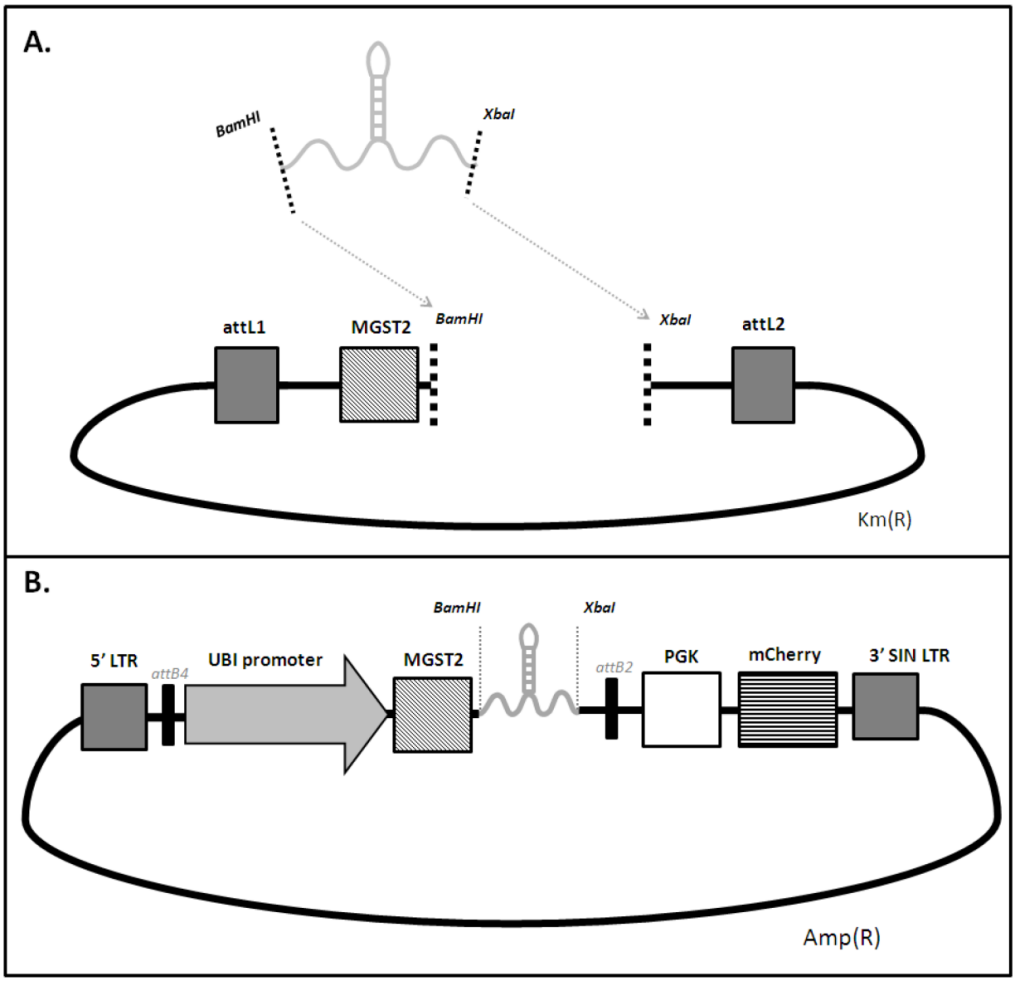
3'-ZAACTATGACTGACATACCTTT

10. Make another wobble on the complementary strand corresponding to position $^{+}$12 of the targeting strand, to suppress abortive processing **As a general rule:** Antiparallel (targeting) sequence being in blue, 100% complimentary to target mRNA (in red)

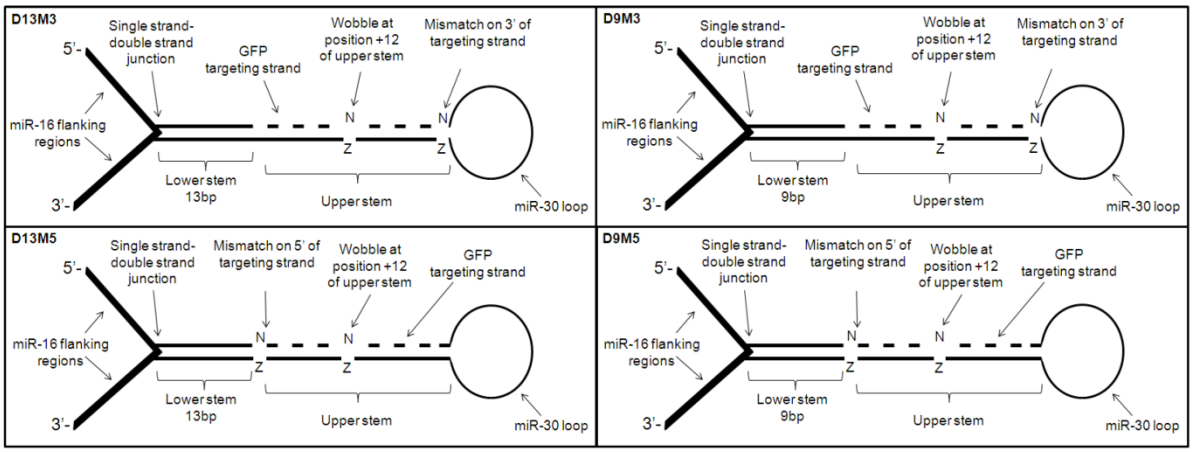
5'- NNNNNNNNNNNNNNNNNNNNN

3'- ZNNNNNNNNNNZNNNNNNNNNN

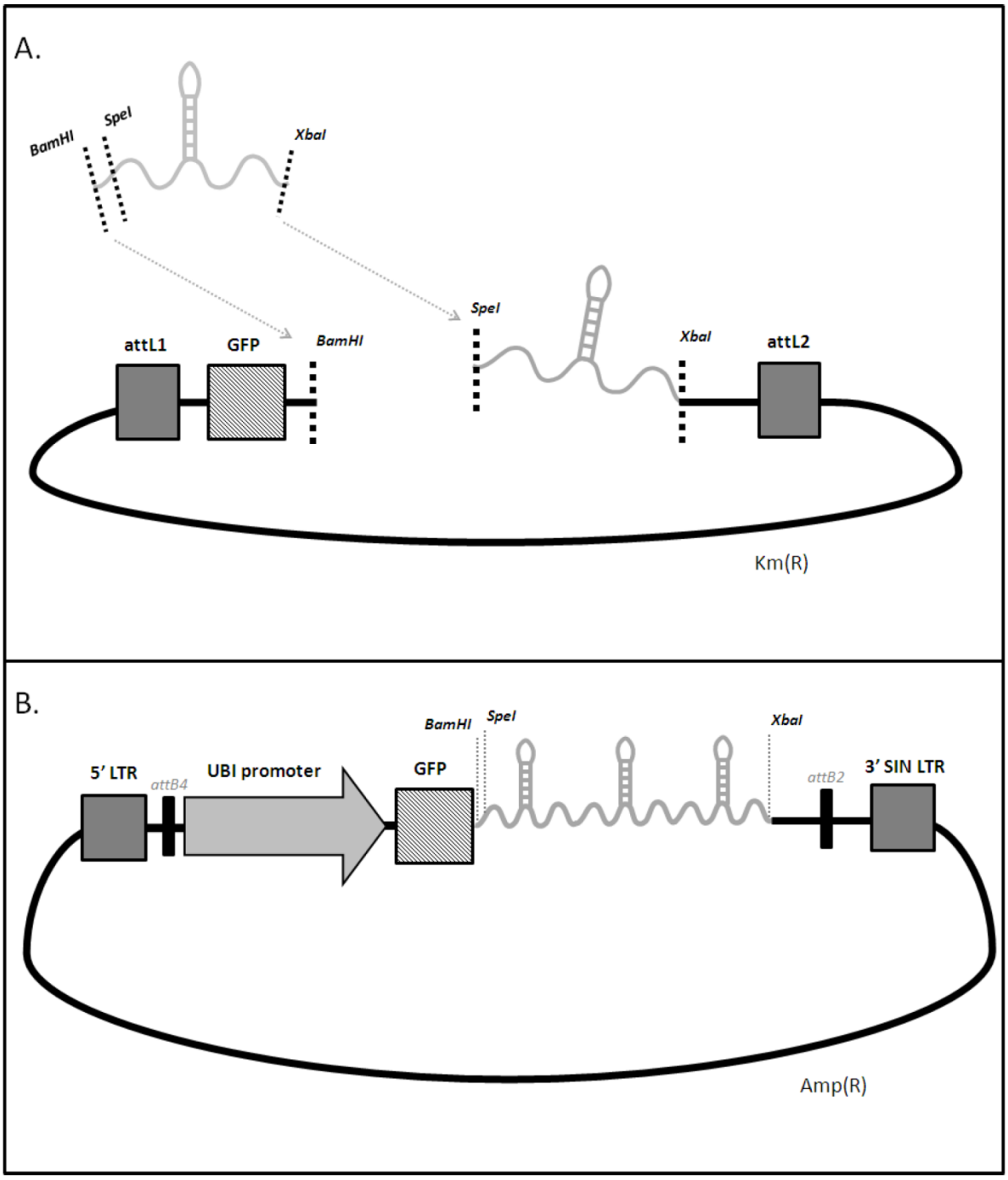
11. Then choose Z to add to bottom oligo according to the N in top oligo facing Z. If N is T, make Z a C. If N is C, make Z a A. If N is A, make Z a C. If N is G, make Z a A
12. Replace NNN stretches by sense and antisense oligos to make the PCR template oligo
13. Amplify this template with the 5' and 3' PCR oligos, as described in Materials and Methods.



Supplementary Figure 1. Schematic diagram of the lentiviral vector plasmid used for the GFP knock-down experiments. (a) A PCR amplicon containing the miRNA hairpin is generated as described in Materials and Methods, cut with BamHI and XbaI restriction enzyme and inserted in a pENTR-L1L2 plasmid downstream of the human MGST2 gene. (b) After LR recombination cloning, the lentivector plasmid contains one cassette expressing the MGST2-miRNA unit under the control of the human Ubiquitin promoter (UBI) and the mCherry living color under the control of the human PGK promoter.



Supplementary Figure 2. Schematic representations GFP knock-down miRNA designs. Four out of the seven anti-GFP miRNA hairpins used in Fig. 1 are depicted here for illustration. The 22 nt segment targeting GFP is on the top strand and is identical in all constructs. Mismatches are generated by altering sequence of the lower passenger strand. The number after D represents the length of the lower stem and the number after M represents the mismatched end of the targeting strand. For example, D13M5 lower stem is 13bp in length and the targeting strand has a mismatch on its 5'. This design is the one named mirGE in this paper.



Supplementary Figure 3. Cloning strategy used to easily clone multiple miRNA hairpins in a lentiviral expression cassette. (a) A pENTR-L1L2 plasmid containing GFP followed by BamHI and XbaI restriction sites is used as recipient for miRNA hairpin cloning (see Materials and Methods section). The first hairpin PCR amplicon (see Materials and Methods section) is cut with BamHI and XbaI and introduced in the pENTR plasmid opened by BamHI and XbaI. Subsequent hairpins are cut by BamHI and XbaI and introduced in front of the previous hairpin using BamHI and SpeI. (b) The final lentiviral plasmid is constructed using LR recombination between a destination lentiviral plasmid (see Materials and Methods section), a pENTR plasmid providing the ubiquitin promoter (UBI) and the pENTR plasmid containing GFP followed by miRNA hairpins.

Supplementary Table 1

(http://www.nature.com/mtna/journal/v3/n10/suppinfo/mtna201458s1.html): Positions of starts and ends of coverage of short RNAs generated by mirGE and miR-30 miRNAs. Sequences from high-throughput sequencing of short RNAs generated from mirGE and miR-30 miRNAs (called reads after illumina®) were aligned to their cognate miRNA sequence (see Figure 4). Results are from cells transduced with lentivector expressing a single hairpin of miR-30 (mir-30_single, 43 % transduced cells), a single hairpin of mirGE (mirGE_single, 23 % transduced cells), or a triple hairpin of mirGE (mirGE_triple, 90 % transduced cells). Numbering after corresponding miRNA sequence is in white horizontal lines. Counts of reads are in yellow horizontal lines. Positions of major starts and ends of reads are indicated in red cells. Orange cells indicate the position of "off-site" reads which are present only in mirGE construct and align to the lower stem. These reads were counted for the score of total reads aligning to miRNA in Supplementary Table 2 and not counted for the score of Drosha cleavage accuracy in Figure 4C.

Supplementary Table 2: Numbers of total reads, reads assigned to miRNAs and reads aligned to miR-30 or mirGE.

Sample	Total number of reads [Mio]	Number of reads that can be assigned to a miRNA* [Mio]	Number of reads that can be assigned to miR-30 or mirGE hairpins	Percentage of reads that can be assigned to miR-30 or mirGE from total miRNA reads	Percentage of targeting strand reads with incorrect start position**
miR-30_Single (Transduction rate 43%)	4.58	2.07	48'506	2.33	7.53
mirGE_Single (Transduction rate 23%)	1.54	0.83	18'282	2.21	0.16
mirGE_Triple (Transduction rate 90%)	5.20	3.78	2'640'128	69.8	0.06
HeLa_R5	4.14	2.34	46***	0.002***	NA

* Total reads were screened to determine number of reads that can be aligned to either endogenous miRNA (mirbase v20) or to introduced constructs miR-30 or mirGE. Untransduced HeLa cells stably expressing CCR5 (HeLa_R5 cells, see Materials and Methods) were used as negative control.

** All reads starting outside of position 72 (miR-30) or position 26 (mirGE) (see Figure 4 A-B) were considered incorrect. The 7.63% incorrect 5' ends of miR-30 is significantly more than the 0.16% incorrect 5' ends of mirGE according to an R prop.test ($p < 2.2e-16$).

***These values were considered as background.

RESEARCH ARTICLE 2

(In review)

Lentivector knock-down of CCR5 in hematopoietic stem cells confers 2 functional and persistent HIV-1 resistance in humanized mice

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Gene-engineered CD34$^+$ hematopoietic stem and progenitor cells (HSPCs) can be used to generate an HIV-1-resistant immune system. However, a certain threshold of transduced HSPCs might be required for an effective therapy, and thus, improved transduction procedures or *in vitro*/*in vivo* selection strategies might be necessary. Here, to obtain a more effective population for transplantation, we combined CCR5 knock-down by a highly efficient miRNA lentivector with pre-transplantation selection of transduced HSPCs. Low-level transduction of HSPCs and subsequent sorting by flow cytometry yielded >70% transduced cells. Mice transplanted with these cells showed functional and persistent resistance to a CCR5-tropic HIV strain: viral load was significantly decreased over months, and human CD4$^+$ T-cells were preserved. In one mouse, viral mutations, resulting presumably in a CXCR4-tropic strain, overcame HIV resistance. Our results suggest that HSPC-based CCR5 knock-down may lead to efficient control of HIV *in vivo.* We overcome a major limitation of previous HIV gene therapy in humanized mice studies in which only a proportion of the cells in chimeric mice *in vivo* are anti-HIV engineered.

Our strategy underlines the promising future of gene engineering HIV-resistant $CD34^+$ cells that produce a constant supply of HIV resistant progeny.

IMPORTANCE

Major issues in experimental long-term *in vivo* HIV gene therapy have been (i) low efficacy of cell transduction at the time of implantation and (ii) transduction resulting in multiple copies of heterologous DNA in target cells. In this study, we demonstrated the efficacy of a transplantation approach with a selection step for transduced cells that allows transplantation of an enriched population of HSPCs expressing a single (low) copy of a CCR5 miRNA. Efficient maintenance of $CD4^+$ T-cells and a low viral titer resulted only when at least 70% of the HIV target cells were genetically modified. These findings imply that clinical protocols of HIV gene therapy require a selective enrichment of genetically targeted cells because positive selection of modified cells is likely to be insufficient below this threshold. This selection approach may not only be beneficial for HIV patients, but also other patients requiring transplantation of genetically modified cells.

INTRODUCTION

Combined anti-retroviral therapy (cART) changed the face of HIV medicine: patients have a life-expectancy close to uninfected people [110]. However, cART has major disadvantages, including adverse events, emergence of drug-resistant strains in patients with poor adherence, a need for life-long intake, psychological dependence, and cost. Thus, cART has not halted the pandemic (http://www.who.int/hiv/en/), and alternative therapies are needed to cure HIV.

Gene therapy has been widely discussed as a possible strategy to cure HIV and has been tested in phase I/II clinical trials. Autologous $CD4^+$ T-cells [111,112] or $CD34^+$ cells [113,114] were gene engineered to express various anti-HIV moieties, including a combination of three RNA-based anti-HIV moieties (tat/rev shRNA, TAR decoy, and CCR5 ribozyme) [113], a tat-vpr-specific anti-HIV ribozyme [114], and a conditionally replicating lentiviral vector expressing long anti-sense to HIV [111] or were gene edited by zinc-finger nucleases for CCR5 knockout [112]. Gene engineering also generated HIV-specific $CD4^+$ or $CD8^+$ T-cells [115,116]. Overall, the effects on HIV infection were modest, but importantly, gene engineering proved to be safe in humans.

The concept of engineering an HIV-resistant immune system received new impetus from the "Berlin patient," who was treated with hematopoietic stem cell transplantation for acute myeloid leukemia. He received bone marrow from a donor homozygous for the Δ32 CCR5 mutation, and thus, the progeny cells did not express CCR5. He was the first documented cure for HIV [44] and provided hope that eliminating CCR5 from the cell surface would be the "Holy Grail" for the cure of HIV. However, another HIV-infected patient suffering from anaplastic large cell lymphoma also received a stem cell transplant from a homozygous CCR5-null donor. Unfortunately, in that case, X4-tropic HIV strains emerged that necessitated the re-initiation of cART [51].

In view of the modest success of phase I/II clinical trials and the data from stem cell transplantation, pre-clinical studies are needed to define the best anti-HIV moieties and the

minimal number of gene engineered cells required to advance gene therapy in HIV. Humanized (hu) mice, which are generated by the transplantation of $CD34^+$ cells, are of particular value in this context. Hu mice excel in their multi-lineage hematopoiesis [117], are highly permissive to HIV [118], and allow for the gene engineering of human $CD34^+$ cells before transplantation [119]. Indeed, various anti-HIV moieties have been investigated in hu mice as gene therapy options, including cellular factors, boosting the anti-HIV-immune response, and the HIV genome itself [119]. These mice were used *in extenso* to investigate the effects of targeting CCR5 by shRNA [55,56,59] or ZNF [54]. All these studies reported a decrease in CCR5 expression in circulating and tissue leukocytes, which were not permissive to HIV *ex vivo*, but only the study by Holt et al. reported a significant decrease of HIV RNA copy number *in vivo* [54]. The other studies either did not analyse the effects on HIV infection *in vivo* [55] or demonstrated no effect on viral load [56]. The results of Holt et al. revealed disruption of CCR5 in only ~20% of all $CD4^+$ T-cells. The follow-up in that study was only 8 weeks. Gene engineering HSPCs with a lentiviral vector encoding the broadly neutralizing anti-HIV human antibody 2G12 showed suppression of HIV RNA but was only studied 7 days after a challenge with a virus containing the corresponding epitope [58]; gene engineering an HIV-specific T-cell receptor also lowered the HIV RNA but only modestly [57]. A very elegant study with $CD34^+$ cells edited with an HIV-1 LTR specific Tre-recombinase showed a potent lowering of HIV RNA activity after HIV challenge in hu mice [120]. All these data are promising; however, we lack a long-term follow-up of the effects of anti-HIV gene therapy in hu mice, the number of gene engineered $CD34^+$ cells needed in the various studies to obtain an HIV RNA lowering effect, and a detailed characterization of the hematopoietic system. A major advance was recently presented by Barclay et al., who purified the gene engineered $CD34^+$ cells via the expression of a truncated version of CD25 [121].

Various means are available for gene engineering $CD34^+$ cells; each has its pros and cons. A great deal of experience exists with shRNA [122]; potential cons may be its potential to trigger the innate immune system [66] and its less than absolute downregulation of the target gene. Targeted gene disruption by ZFN, Talen or Crispr/cas has the advantage of complete disruption of the

gene of interest [123-125]; however the modest rate of gene engineering CD34+ cells [126], the potential of off-target effects [126-128] and the lack of clinical experience represent substantial hurdles for wider use *in vivo*.

We recently reported a novel microRNA-based gene knock-down strategy with improved knock-down, relative to methods conventionally used [129]. A triple hairpin cassette targeting CCR5 resulted in >90% CCR5 knock-down upon single-copy transduction in HeLa cells. The aim of the present study was to assess whether gene engineering $CD34^+$ cells with this vector construct results in down-regulation of CCR5 in progeny cells in hu mice and whether it could protect against HIV challenge *ex vivo* as well as *in vivo*. Since some evidence in the literature exists that the number of gene engineered $CD34^+$ cells is a major determinant of the success of the anti-HIV moieties [54,56], we also made a major effort to generate hu mice with a very high number of gene engineered $CD34^+$ cells. Finally, we characterised *in extenso* the hematopoietic system subsequent to HIV infection in hu mice with gene-engineered $CD34^+$ cells.

RESULTS

Transplanting CD34+ cells with partial CCR5 knock-down does not hinder HIV replication.

We previously developed a highly efficient microRNA called "mirGE" (30) that allows efficient knock-down by single-copy transduction (Fig. S1a). Here we explore the potential of a mirGE lentivector targeting CCR5 to produce an HIV-resistant immune system in hu mice. The construct consists of a triple hairpin, and the vector cassette contains GFP driven by the same promoter as the miRNA that allows transduced cells to be identified directly. To minimize possible cellular perturbations from multiple vector inserts, we established a protocol that gave us a transduction rate of 20–30%. This transduction rate was based on previous work and should correlate with single-copy integration [89]. In a first series of experiments, mice were transplanted with mirGE-transduced $CD34^+$ cells without further manipulation (R5 knock-down mice); the $CD34^+$ cells were a mixture of transduced (20–30%) and untransduced (70–80%) cells (data not shown). As controls, we used either mice transplanted with CD34+ cells transduced with a control GFP lentivector (control-transduced mice; Fig. S1b) or with untransduced $CD34^+$ cells (untransduced mice). Upon infection with a R5-tropic HIV (YU-2), the percentage and absolute number of GFP-positive $CD4^+$ T-cells were increased in R5 knock-down cohorts and not in control-transduced cohorts (Fig. 1a, and Fig. 3g and h). The CD4+ cell population, however, remained the same over the observation period of 92 days (cohort 1), or showed a CD4+ T-cell loss only at day 134 days (cohort 2) (Fig. 1b). We explain this increase in GFP+ HIV-resistant CD4+ cells as the result of preferential expansion at the cost of untransduced CD4+ T-cells, while the lymphoid system tries to keep the lymphoid T-cell number constant. However, HIV replication was similar in R5 knock-down mice and control-transduced mice over the observation period at 92 and 134 days (Fig. 1c). Analysis of absolute numbers of $CD4^+$ T-cells (cohort 2) indicated that the control-transduced mice lost $CD4^+$ T-cells; whereas, they were more or less maintained in the R5 knock-down mice (data not shown).

In the R5 knock-down mice, CCR5 was down-regulated in the GFP-positive $CD4^+$ T-cells, but CCR5 was detected on the GFP-negative $CD4^+$ T-cells in blood and spleen (Fig. 1d and Fig. S2). In the control-transduced mice, CCR5 was detected on GFP-negative and -positive $CD4^+$ T-cells. We verified the efficacy of our gene engineering approach by separating transduced from untransduced splenocytes (R5 knock-down mice) by FACS and infected the populations *ex vivo* with R5 tropic HIV. GFP-positive splenocytes had no HIV replication (Fig. 1e).

These results suggest that CCR5 knock-down efficiently protects $CD4^+$ T-cells from HIV infection, while CCR5-expressing $CD4^+$ T-cells are eradicated. In our mice, despite HIV challenge, at least a proportion of the ~70–80% untransduced hematopoietic stem cells survive and continue to produce HIV-permissive $CD4^+$ T-cells, which sustains high HIV titers. It is still not entirely clear what effect HIV infection has on $CD34^+$ cells and to what extent they are depleted, if at all [130].

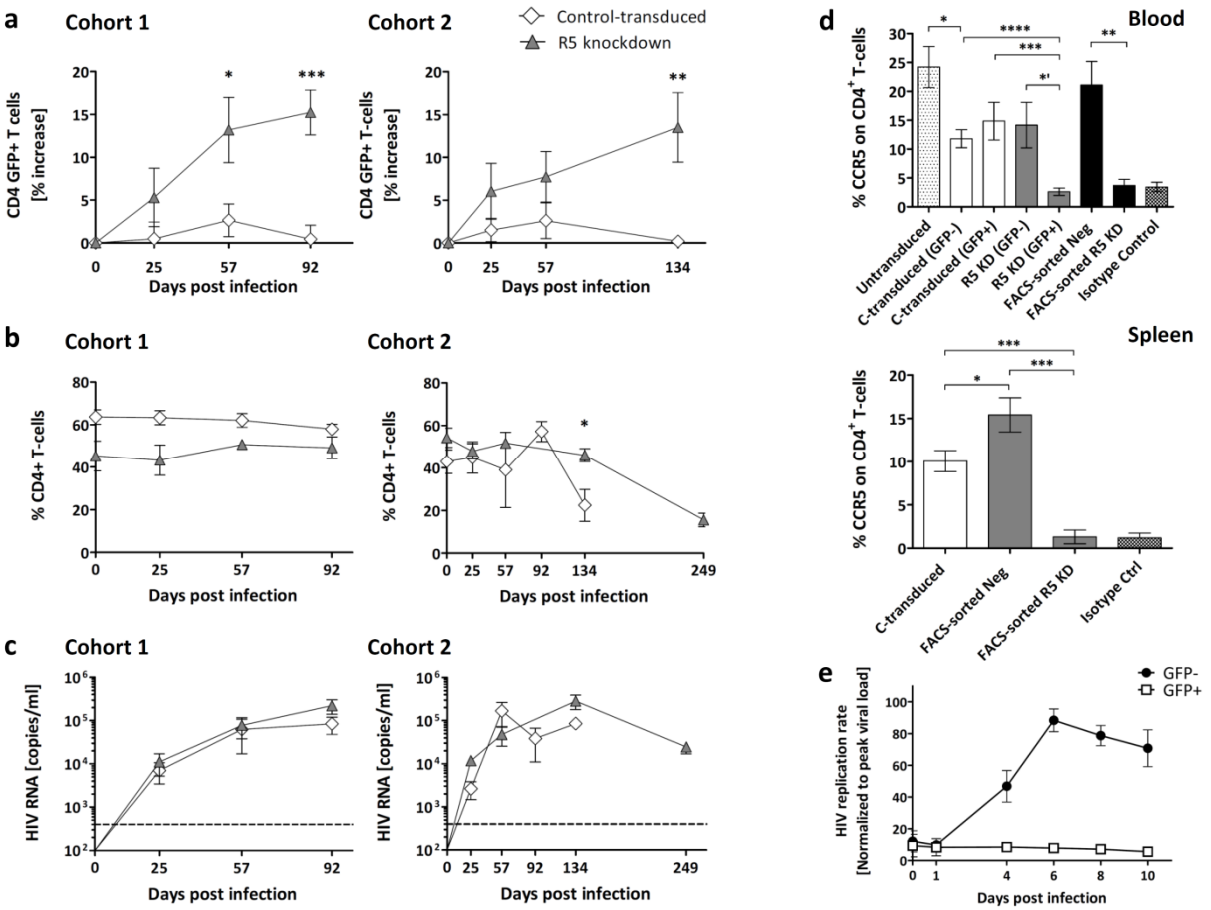


Figure 1. Homeostatic expansion of GFP-positive CD4+ T-cells in R5 knock-down mice despite sustained R5-tropic (YU-2) HIV infection. (a) The percentage change of GFP-positive CD4+ T-cells in the peripheral blood of control-transduced and R5 knock-down mice. Control-transduced mice (Cohort 1, n=8; Cohort 2, n=4) and R5 knock-down mice (Cohort 1, n=6; Cohort 2, n=5). Mean ± sem.; *P = 0.0152, **P = 0.0034, ***P =0.0002. P values determined by two-tailed unpaired t-test. (b) HIV RNA copies per ml of blood plasma collected for the control-transduced and R5 knock-down mice cohort 1 and cohort 2 over 92 and 134 days, respectively. Time of termination was chosen at random for the various groups. The dashed line indicates 400 copies/ml, detection limit of the HIV RNA assay. Mean ± sem. (c) Frequency of total CD4+ T-cells (percentage of total CD3 T-cells) for the control-transduced and R5 knock-down cohorts 1 and 2. Mean ± sem.; *P = 0.0146. P values determined by two-tailed unpaired t-test. (d) Percentage CCR5 expression on total CD4+ T-cells in peripheral blood and spleen of various cohorts of mice. Blood: mean ± sem .; *P = 0.0237, *'P = 0.0121, **P = 0.0035, ***P = 0.0007, ****P = 0.0001. Spleen: mean ± sem.; *P = 0.041, ***P = 0.0003. P values determined by two-tailed unpaired t-test. (e) HIV replication is inhibited ex vivo in GFP-positive sorted splenocytes from R5 knock-down mice. Splenocytes were isolated from R5 knock-down mice 20 weeks after CD34+ cell injection. Splenocytes were sorted into GFP-positive (n=5) and GFP-negative (n=5) fractions at 99% purity and challenged with R5 tropic HIV (YU-2). The amount of HIV production in the

culture supernatant was monitored by HIV p24 ELISA. Viral loads were normalized to the peak viral load in each condition (mean ± sem).

Transplantation of purified CCR5 knock-down CD34$^+$ cells results in mice with "pure" populations of transduced cells *in vivo.*

The lack of resistance to HIV infection was likely due to the chimerism of transduced and untransduced CD34$^+$ cells in our initial experiments. Therefore, we sorted the CD34$^+$ cells after transduction into CCR5 knock-down, GFP-positive and -negative fractions obtaining a >90% pure population of GFP-positive CD34$^+$ cells (Fig. S3a-d). Mice transplanted with GFP-sorted cells were called "FACS-sorted R5 knock-down mice." Analysis of the peripheral blood in six of the FACS-sorted R5 knock-down animals showed a single GFP-positive peak for human CD45$^+$ and CD4$^+$ T-cells, suggesting that only transduced CD34$^+$ cells engrafted in these mice (Fig. 2); the level of GFP-positive cells was a major criterion for successful gene engineering and engraftment. Mice transplanted with the GFP-negative fraction were called "FACS-sorted negative mice." They developed CD4$^+$ and CD8$^+$ T-cell populations with no GFP expression (Fig. 2). In contrast, the control-transduced mice had two distinct GFP-negative and -positive populations for CD45$^+$ and CD4$^+$ T-cells (Fig. 2). A summary of mice used in this study is indicated in (Table 1). Mice with less than 5% human engraftment in the peripheral blood before HIV infection were excluded.

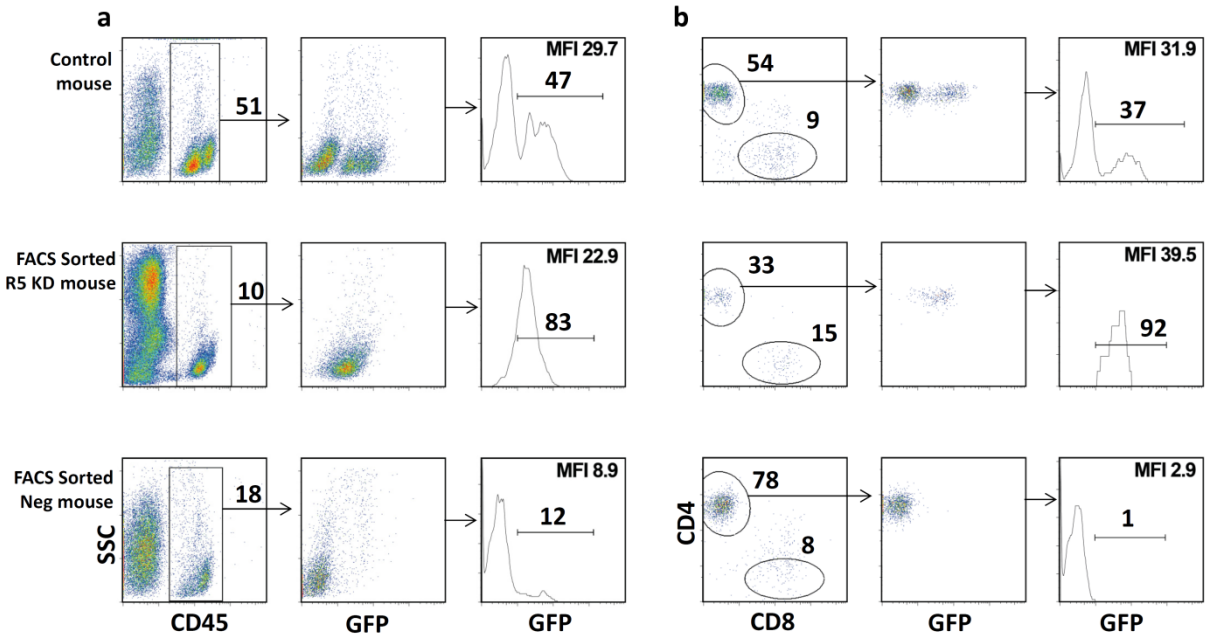


Figure 2. GFP-positive CCR5 knock-down sorted CD34⁺ HSPCs produced mice with "pure" populations of transduced cells *in vivo*. (a) FACS plots showing the percentage human engraftment CD45⁺ (percentage of live cells) and GFP-positive CD45⁺ (percentage of human CD45) cells for representative control-transduced, FACS-sorted R5 knock-down and FACS-sorted negative mice before HIV infection. (b) FACS plots showing the percentage CD4⁺ T-cells (percentage of CD3⁺), CD8⁺ (percentage of CD3⁺) and GFP-positive CD4⁺ T-cells (percentage of CD4⁺) in the peripheral blood before HIV infection of the same mice as in *(a).* For all cell subset analysis, the sub-gating was done as follows, total live cells, CD45⁺, CD3⁺, CD4⁺, CD8⁺. Mean fluorescence intensity (MFI) for GFP is indicated.

Table 1: Mice used in the study.

			Number of mice with			
	%GFP⁺ CD34⁺ cells; [Pre-transplantation]	**Total mice**	**% Engraftment (>5% hCD45⁺)**	**% GFP⁺CD4⁺ T-cells (>70%)**	**% GFP⁺CD4⁺ T-cells (20-70%)**	**% GFP⁺CD4⁺ T-cells (<20%)**
Untransduced	n/a	15	11	n/a	n/a	n/a
Control-transduced	15-40	17	12	0	5	7
R5 knock-down	17-20	15	11	0	0	11
FACS GFP-positive	12-32	20	10	6	4	0
FACS GFP-negative	n/a	26	15	n/a	n/a	n/a

Transplanting purified CCR5 knock-down CD34$^+$ cells dramatically lowered viral load and protected HIV target cells *in vivo*.

The FACS-sorted R5 knock-down mice had markedly lower viral loads than the FACS-sorted negative mice over 28 weeks (Fig. 3a). Peak viremia for the FACS-sorted R5 knock-down mice was on average $4.2x10^3$ copies/ml, and FACS-sorted negative mice had $3.5x10^5$ copies/ml. Viral loads for the untransduced, control-transduced, R5 knock-down, and FACS-sorted negative mice were similar (Fig. S4a). The FACS-sorted R5 knock-down mice had lower viral loads than all cohorts (Fig. S4b).

CD4$^+$ T-cells (percentage of total CD3$^+$ T-cells) from the FACS-sorted negative mice declined steadily upon infection (day 0: 55%; day 134: 20%) (Fig. 3b). In contrast, the FACS-sorted R5 knock-down mice showed a steady increase in CD4$^+$ T-cells (day 0: 33%; day 196: 65%). Furthermore, the absolute numbers of CD4$^+$ T-cells increased for the FACS-sorted R5 knock-down mice but decreased for the FACS-sorted negative mice (data not shown).

At euthanasia, for the FACS-sorted R5 knock-down mice, 70% of CD4$^+$ T-cells in the blood and 83% of CD4$^+$ T-cells in the spleen were GFP-positive (Fig. 3c-d). In the control-transduced and R5 knock-down groups, this was on average 20% and 18% in the blood and 21% and 20% in the spleen, respectively (Fig. 3c,d). Similarly, the CD4$^+$/CD8$^+$ T-cell ratios in the blood and spleen (end/pre-infection) were very low in the various groups except for the FACS-sorted R5 knock-down group (Fig. 3e,f). Absolute numbers of GFP-positive CD4$^+$ T-cells expanded significantly upon HIV challenge in the FACS-sorted R5 KD mice (Fig. 3i), and CCR5 expression was down-regulated in the blood and spleen of the FACS–sorted R5 knock-down mice (Fig. 1d and Fig. S5).

In summary, transplantation of GFP-positive sorted CD34$^+$ cells produced mice with high levels of gene-engineered CCR5 knock-down CD4$^+$ T-cells *in vivo*. This resulted in long-term inhibition of HIV replication *in vivo* and preservation of HIV target cells in the blood and spleen.

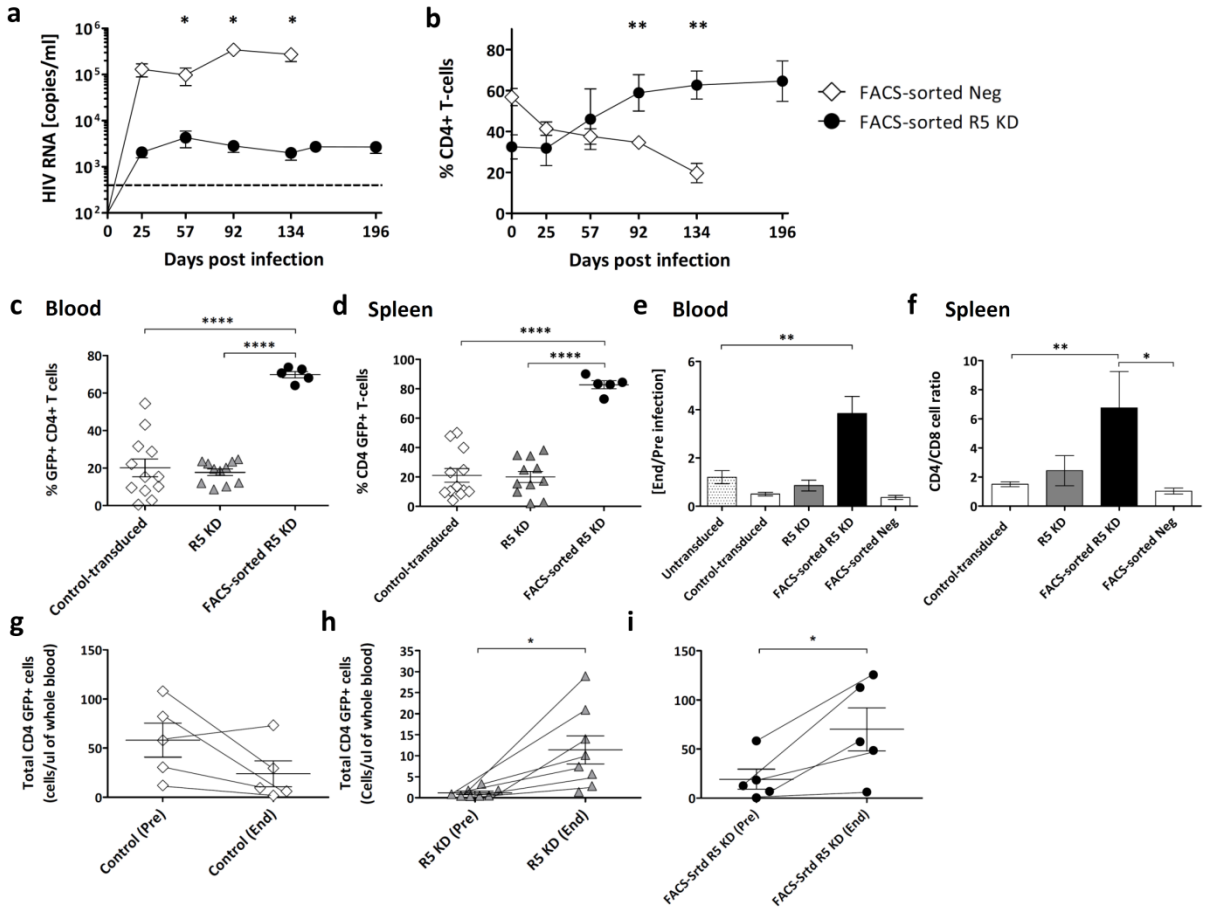


Figure 3. Sustained HIV viral load inhibition in FACS-sorted R5 knock-down mice. (a) HIV RNA copies per ml in blood plasma from FACS-sorted R5 knock-down n=5 (YU-2) and FACS-sorted negative n=15 (YU-2 n=9 or JRCSF n=6) mice collected over 134 and 196 days, respectively. The viral load detection limit is indicated by the dashed line (400 copies/ml). Mean ± sem.; Day 57 *P = 0.0486, day 92 *P = 0.0188, and day 134 *P = 0.0391. P values determined by a two-tailed unpaired t-test. (b) Percentage $CD4^+$ T-cells of FACS-sorted R5 knock-down (n=5) and FACS-sorted negative (n=15) mice. Mean ± sem.; Day 92 **P = 0.0017 and day 134 **P = 0.0018. P values determined by two-tailed unpaired t-test. (c) Percentage GFP-positive $CD4^+$ T-cells in the peripheral blood at termination. Mean ± sem.; ****P = 0.0001. P values determined by two-tailed unpaired t-test. (d) Percentage GFP-positive $CD4^+$ T-cells in the spleen at termination. Mean ± sem.; ****P = 0.0001. P values determined by two-tailed unpaired t-test. (e) Change of $CD4^+/CD8^+$ T-cell ratio in the peripheral blood, comparing the $CD4^+/CD8^+$ T-cell ratio of each cohort end/pre-infection. Mean ± sem.; **P = 0.0026. P values determined by two-tailed unpaired t-test. (f) $CD4^+/CD8^+$ T-cell ratio at termination in the spleen of various cohorts of mice. Mean ± sem.; **P = 0.0059, *P = 0.039. P values determined by two-tailed unpaired t-test. (g-i) Absolute numbers of GFP^+ $CD4^+$ T-cells or total $CD4^+$ T-cells/µl of blood of representative mice from the control-transduced (n=5), R5 knock-down (n=8) and FACS-sorted R5 knock-down (n=5) cohorts. Mean ± sem.; *P = 0.0194, *P = 0.0369, respectively. P values determined by paired t-test.

Outlier analysis and follow-up of mice that did not meet acceptance criteria.

The FACS-sorted R5 knock-down mice typically controlled the virus long-term (titers < 10^4 copies/ml) while maintaining a high level of GFP-positive $CD4^+$ T-cells in the blood (Fig. 4a). In contrast, a single FACS-sorted R5 knock-down mouse (#954) showed an unexpected decline in GFP-positive $CD4^+$ T-cells with high HIV copy numbers, >10^5 copies/ml on days 57 and 92 (Fig. 4a). We performed an outlier analysis (see methods section), and at four different times, viral loads detected in animal #954 were statistical outliers (Fig. 4a). Based on this, animal #954 was not included in the mean values and was analyzed separately (see below). At termination, this mouse had much lower splenic engraftment of total human splenocytes with 31% human cells (Fig. S6a,b) compared to the FACS-sorted R5 knock-down mice which had 54% (mean ± sem: 11%). GFP-positive $CD4^+$ T-cells were barely detected in the spleen of mouse #954 (6%), while the FACS-sorted R5 knock-down mice had, on average, 83% (mean sem ± 2%) GFP-positive $CD4^+$ T-cells in the spleen (Fig. 3d and Fig. S6a,b).

The inclusion criterion we defined as successful reconstitution for FACS-sorted R5 knock-down mice was 70% GFP^+ $CD4^+$ T-cells in the peripheral blood before infection. Four mice did not meet these criteria despite being transplanted with GFP-positive sorted $CD34^+$ cells (Fig. 4b). Mouse #1113 had no protection against HIV and had a limited expansion of GFP-positive $CD4^+$ T-cells (23% on day 0 to 43% on day 137). Mice #958 and #1115 had massive expansions of GFP-positive cells reaching close to 100% of the total $CD4^+$ T-cell population which went in parallel with a decrease in the viral load (Fig. 4b, and Fig. S7a,b). For mouse #1608, the dynamics of GFP-positive $CD4^+$ T-cell recovery and viral load were slower, and GFP-positive $CD4^+$ T-cell recovery on day 134 was less extensive. However, the GFP-positive $CD4^+$ T-cells had increased substantially from 36% at (day 0) to 63% at day 60 post-infection (Fig. 4b). These three mice (#958, #1115, #1608) maintained a high CD4/CD8 ratio, as well as high percentage of GFP-positive $CD4^+$ T-cells in the blood and spleen (Fig. S7a-c).

Strikingly, we observed this level of expansion (Fig. 4b and Fig. S7) of HIV-resistant GFP-positive $CD4^+$ T-cells cells and a concomitant inhibitory effect on HIV only in mice transplanted with CCR5 knock-down GFP-sorted $CD34^+$ cells (these animals had 30±4% GFP^+ $CD4^+$ T-cells on day 0). This degree of expansion was not seen in R5 knock-down mice (which had 5±2% GFP^+ $CD4^+$ T-cells before HIV infection). Based on these results, we estimate that at least 20% of $CD4^+$ T-cells need to be CCR5 repressed to observe homeostatic expansion and relevant effects on viremia.

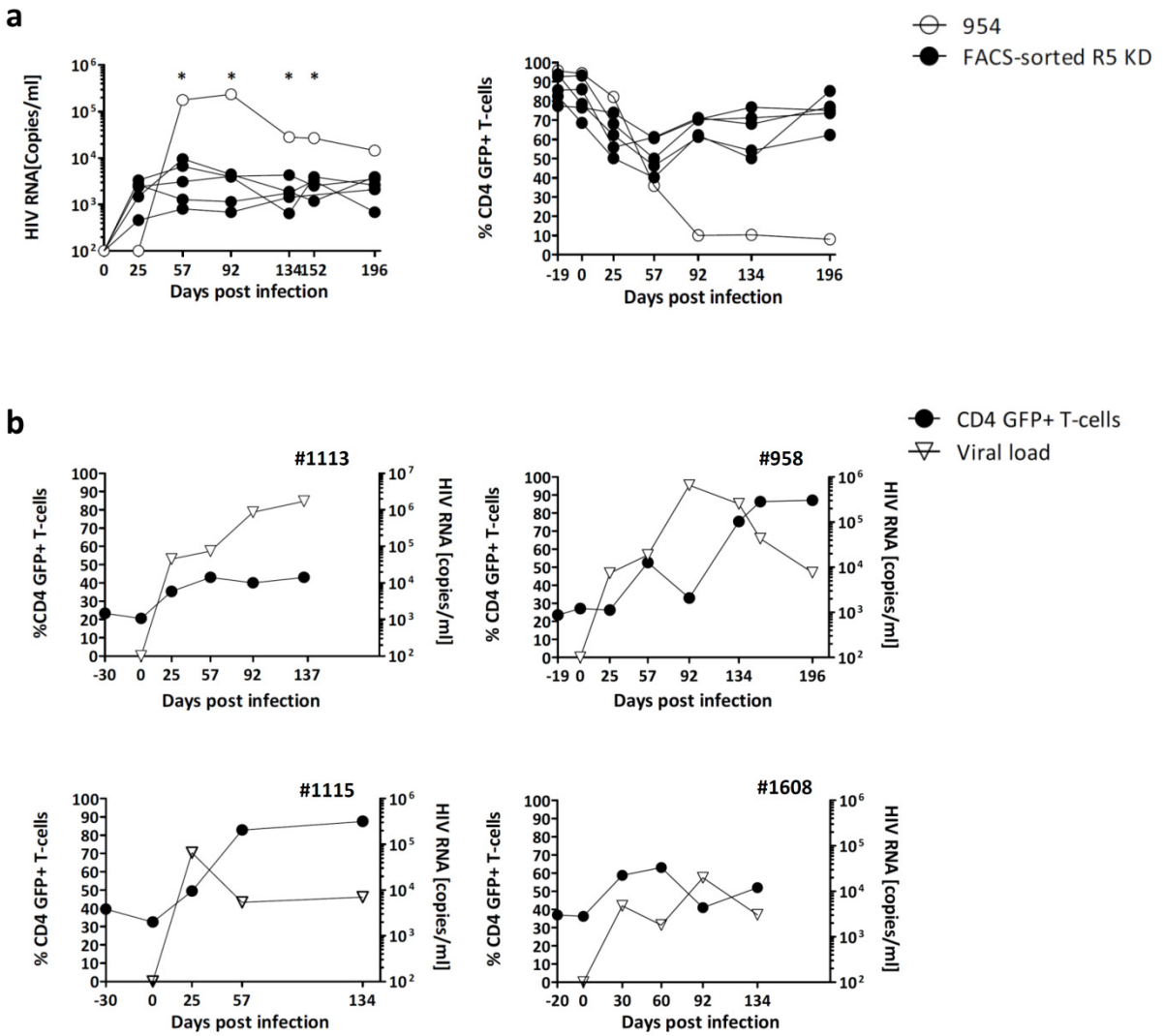


Figure 4. Outlier analysis and examples of viral load control due to homeostatic expansion of transduced cells. (a) Left panel, viral load of the FACS-sorted R5 knock-down mice (YU-2) and viral load of "outlier FACS-sorted R5 knock-down mouse #954" (YU-2). Right panel, percentage GFP-positive $CD4^+$ T-cells of the FACS-sorted R5 knock-down mice and mouse #954. Percentage GFP-positive $CD4^+$ T-cells as a percentage of total $CD4^+$ T-cells. *P* values determined by GraphPad Outlier calculator. *$P < 0.001$. (b) Viral load and percentage GFP-positive $CD4^+$ T-cells of three individual FACS-sorted R5 knock-down mice (mouse #1113, JRCSF, #958, YU-2, #1115, JRCSF and #1608, JRCSF) that were excluded due to not reaching the inclusion criteria of over 70% GFP-positive $CD4^+$ T-cells before HIV infection.

Preserved engraftment and preferential expansion of central memory T-cells in FACS-sorted R5 knock-down mice upon HIV infection.

Engraftment as reflected in peripheral blood decreased in all control cohorts but increased in the FACS-sorted R5 knock-down mice (Fig. 5a). This effect on total engraftment was even more impressive in the spleen. FACS-sorted R5 knock-down mice had 10 times more human cells than control cohorts (Fig. 5b).

We evaluated the $CD4^+$ and $CD8^+$ effector ($CD45RA^{pos}$; $CCR7^{neg}$), effector memory ($CD45RA^{neg}$; $CCR7^{neg}$), naive ($CD45RA^{pos}$; $CCR7^{pos}$) and central memory ($CD45RA^{neg}$; $CCR7^{pos}$) T-cell subsets in the blood and spleen of the FACS-sorted R5 knock-down and representative FACS-sorted negative mice (Fig. 5c-f). In the peripheral blood of the FACS-sorted negative mice, the frequency of central memory $CD4^+$ T-cells was significantly decreased, and the $CD8^+$ central memory T-cell subset was unchanged (Fig. 5c,e). In contrast, central memory $CD4^+$ and $CD8^+$ T-cells were increased in the FACS-sorted R5 knock-down mice. Similarly, more $CD4^+$ and $CD8^+$ central memory T-cells were present in the spleens of the FACS-sorted R5 knock-down mice (Fig. 5d,f). We observed no differences between the cohorts for effector and effector memory cells (data not shown). Notably, however, there was a trend towards a decrease in naive $CD4^+$ and $CD8^+$ T-cells in all cohorts (data not shown).

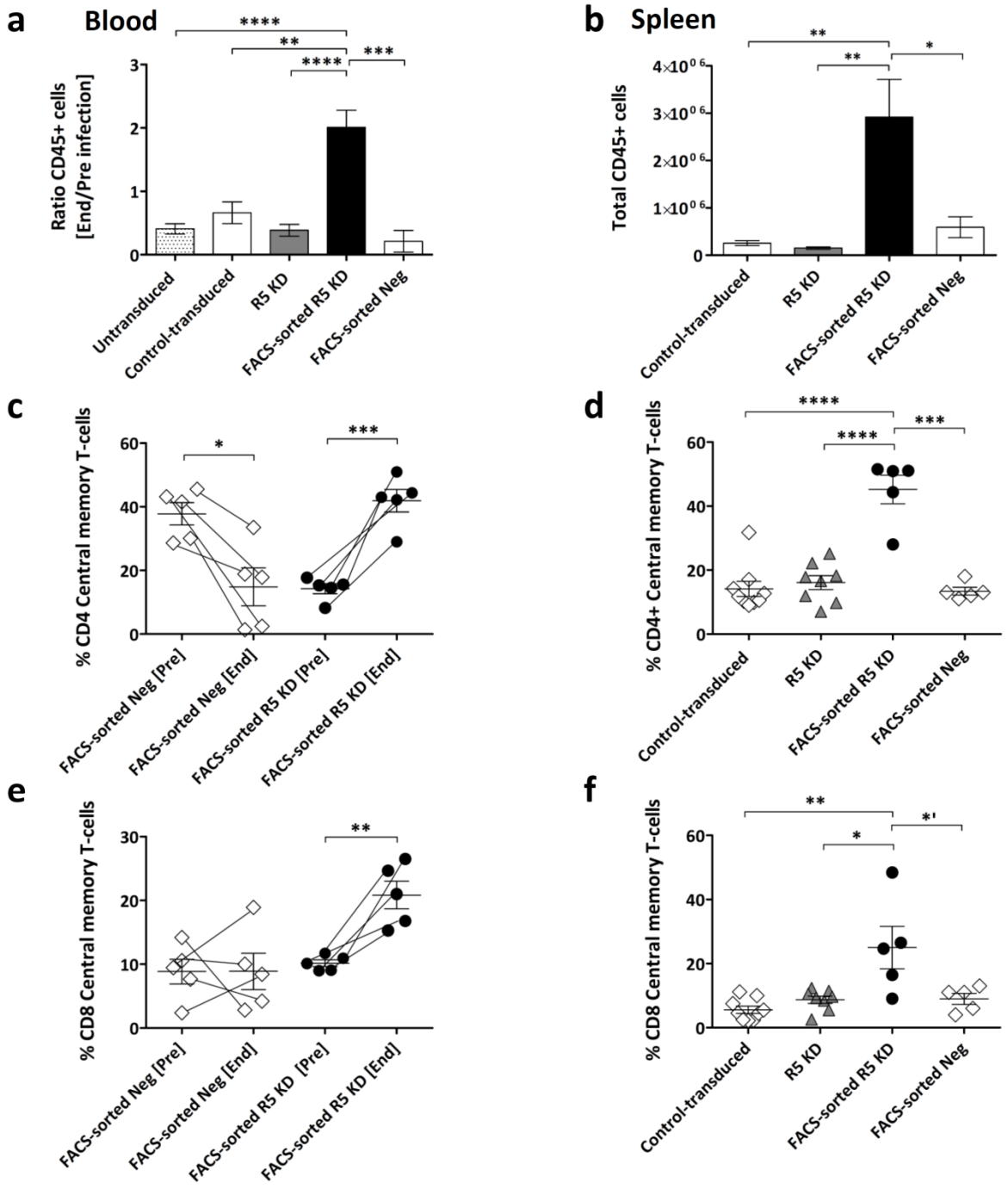


Figure 5. Increased engraftment and central memory T-cells in blood and spleen of FACS-sorted R5 knock-down mice. (a) Change in level of peripheral blood engraftment expressed as the ratio of total CD45+ end/pre-infection. Mean ± sem.; ***P* = 0.0031, ****P* = 0.0006, *****P* < *0.0001. P* values determined by two-tailed unpaired *t*-test. (b) Absolute numbers of human cells (CD45+) in the spleen at termination. Mean ± sem.; **P = 0.0229,* ***P* < 0.0019*. P* values determined by two-tailed unpaired *t*-test. (c,e) Percentage of $CD4^+$ and $CD8^+$ central memory T-cells in the peripheral blood, pre-infection and at the end time point. Mean, sem.; **P = 0.0153, **P = 0.0042, ***P = 0.0004. P* values determined by paired *t*-test. (d,f) Percentage $CD4^+$ and $CD8^+$ central memory T-cells in the spleen at termination. Mean ± sem.; *'*P* = 0.0472, **P* = 0.0106, ***P* = 0.0022, ****P* = 0.0001, *****P* < 0.0001. *P* values determined by two-tailed unpaired *t*-test.

Evidence of shift from R5 to X4 tropic strain in one mouse.

As described above (Fig. 4), FACS-sorted R5 knock-down mice had a low viral load and maintained high levels of CCR5 knock-down $CD4^+$ T-cells. Mouse #954 was clearly an outlier: it had high viral titers and lost GFP-positive $CD4^+$ T-cells (Fig 6a). We hypothesized that this mouse might have had a tropism shift of the virus from R5 to X4. We performed HIV population sequencing (from plasma) on days 57 and 196. As a control, we analyzed mouse #958 (Fig. 6b and Fig. S7a). Sequencing revealed no mutations in mouse #958. Mouse #954 had mutations in the V3 loop of the HIV envelope sequence (Fig. 6c), resulting in amino acid substitutions to basic amino acids as indicated (Fig. 6d). Substitutions in the V3 loop to basic amino acids have been reported to result in a switch from R5 to X4 tropism [131].

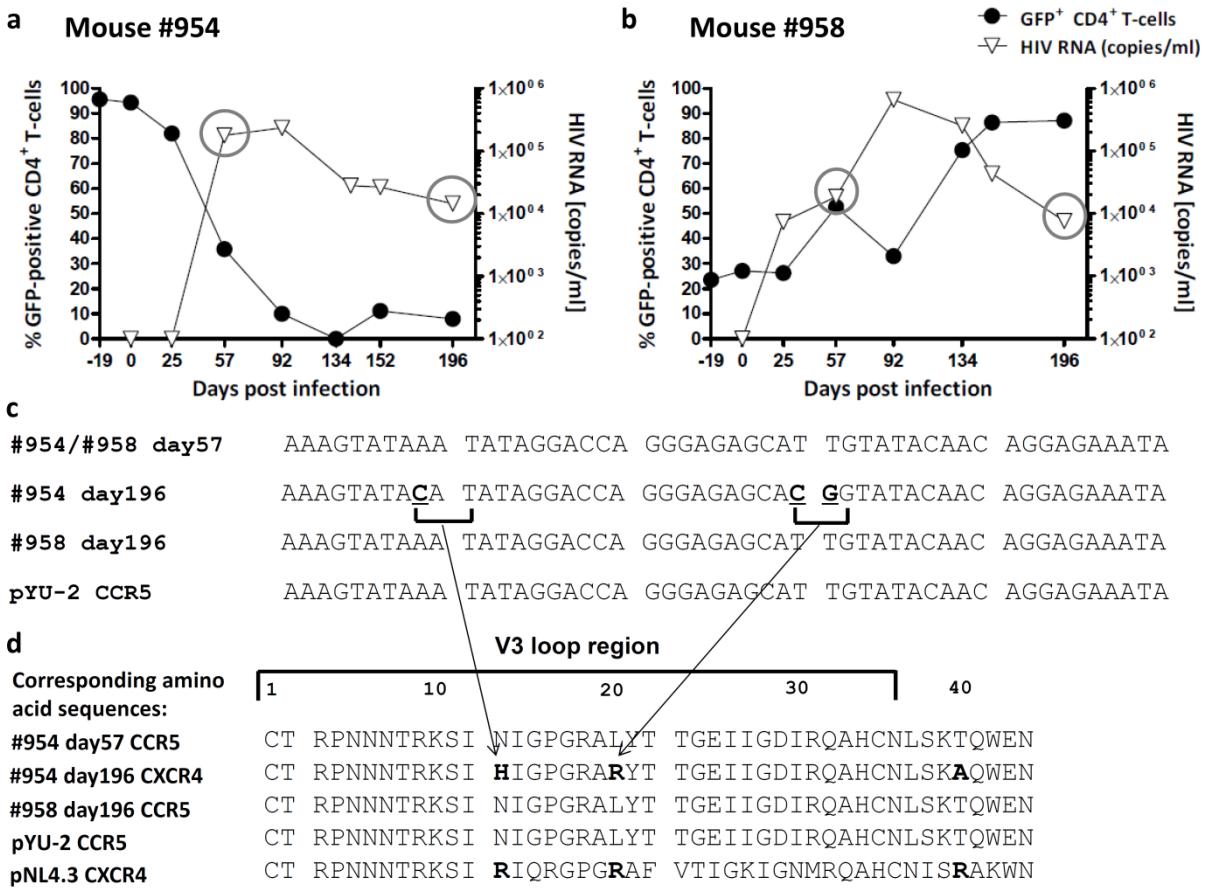


Figure 6. Population sequencing of HIV in plasma: evidence for gene-therapy failure. (a,b) Plots showing the percentage of GFP-positive CD4+ T-cells in peripheral blood (as a percentage of CD3+ T-cells) on the left Y axis and HIV viral load on the right Y axis for mice #954 and #958, respectively. Mouse #954 had a high and sustained viral load overtime with a complete loss of GFP-positive CD4+ T-cells. Mouse #958 experienced an expansion of GFP-positive CD4+ T-cells from less than 30% on day 19, to over 80% on day 196. (c) On day 57, both animals had a homogenous HIV population in the peripheral blood, sequencing data of HIV envelope V3 loop is consistent with an R5 tropic HIV strain. On day 196, animal #954, which experienced a complete loss of GFP-positive CD4+ T-cells (blood and spleen), had detectable mutations within the V3 loop of HIV. For mouse #958, no mutations were detected in the V3 loop on day 196, indicating the presence of a homogenous HIV population. (d) Amino acid changes to basic amino acids H and R. According to a Geno2Pheno (http://www.geno2pheno.org/) analysis of the obtained sequence in *(c)*, there is only 18% confidence that the virus at day 196 of mouse #954 was not an X4 variant.

DISCUSSION

Here we investigated a lentiviral vector–based, CCR5-targeting miRNA as a tool for engineering an HIV-resistant human immune system. We show that i) the miRNA-based vector was very efficient in down-regulating CCR5 from T-cells and prevented their infection by HIV *ex vivo*, ii) only mice that were transplanted with a pre-selected population of transduced $CD34^+$ cells and maintained gene engineered $CD4^+$ T-cells had a dramatically reduced viral load (functional cure), and iii) the HIV-infected mice transplanted with miRNA CCR5 gene-engineered CD34+ cells showed a dramatic expansion of memory T-cells (i.e., the miRNA edited T-cells were mainly of this phenotype). Thus, we provide here pre-clinical proof-of-concept for gene engineering of an HIV-resistant immune system through the use of vector-mediated miRNA expression and the need for a certain threshold of gene engineered CD34+ cells for functional cure of HIV.

While gene engineering HIV-resistant cells is a viable option to cure of HIV, major issues remain to be solved. These include finding the best anti-viral moiety or combination, the most efficacious way to gene engineer the CD34+ cells, and the threshold of gene engineered CD34+ cells needed for functional cure.

Lentivirus-based transduction has been supplemented by gene-targeting methods, such as ZNF- or Talen nucleases, or the Crispr/Cas system for gene editing [123-125]. However, these methods are still in their infancy, and off-target effects are unknown. Gene engineering in primary cells is only modestly effective, and even though no adverse events have been reported, there is less experience in clinical trials than with lentivirus-based transduction. Thus, we opted for lentivirus-based gene engineering [132-134]. Furthermore, we are the first to engage in miRNA technology for knocking down the HIV co-receptor CCR5 in CD4+ T-cells via gene engineering CD34+ cells. miRNAs closely mimic naturally occurring pri-miRNAs and, thus, are less likely to cause oversaturation of the RNA interference pathway and to affect cellular homeostasis than the widely used shRNAs [63,65]. However, miRNAs are thought to have a lower capacity to down-regulate target genes than shRNAs. Here we used a miRNA we developed with optimized

features that efficiently knock-down target genes upon single-copy vector transduction [129]. *Ex vivo* sorted cells were indeed resistant to a challenge with CCR5-tropic strains. However, bulk transplantation of transduced CD34+ cells into mice resulted in a human lymphoid system that replicated HIV similar to untransduced hu mice but preserved CD4+ T-cell counts. Similar data have been reported [56].

We hypothesized that a threshold of gene engineered cells would be required to see an effect on the HIV load; otherwise, the HIV permissive CD4+ T-cells that originated from the untransduced CD34+ cells would "outnumber" the HIV-resistant cells. Thus, we used GFP to allow for efficient sorting of transduced $CD34^+$ cells before transplantation. By doing so, we found that, to achieve long-term suppression of viral load, more than 70% of CD34+ transplanted should be gene engineered. Walker et al. obtained an average engraftment (±STD) level of anti-HIV vector transduced cells of 17.5±8% in the peripheral blood and argued that these numbers of cells were insufficient to see any decrease in plasma viremia [56]. Furthermore, a very recent publication sorted the gene engineered CD34+ cells with a truncated version of CD25 before their transplantation into 2–5-day-old NRG mice [121]. They found that mice transplanted with tCD25 purified CD34+ cells had normal multi-lineage hematopoiesis similar to mice transplanted with untransduced CD34+ cells. Upon HIV challenge, tCD25 transplanted mice did not suffer from HIV induced CD4+ T-cell depletion as did the untransduced mice and tCD25 mice had a 1.5 log inhibition in plasma viremia, compared to mice with untransduced CD34+ cells. Our data nicely complement the data provided by Walker et al. and Barclay et al. and underline the importance of the number of transduced cells that are required for efficient HIV gene therapy. Notably, two hu mice transplanted with purified gene engineered $CD34^+$ cells showed at baseline 23 and 40% GFP^+ cells which expanded substantially upon HIV infection; the expansion went along with a decrease in viral load. These two mice were reminiscent of the data reported by Holt et al where disruption of CCR5 by zinc-finger nucleases was achieved in ~20% of $CD34^+$ cells and resulted in viral repression over time [54].

Obviously, in humans, GFP-based sorting would not be an option, given the xenogeneic nature of the protein. However, novel strategies for sorting of transduced $CD34^+$ cells based on the expression of truncated cellular surface receptors, such as CD25 [121], the epidermal growth factor receptor [135] or the nerve growth factor receptor [136], are very promising for achieving high numbers of engrafted gene engineered cells. An alternative approach to pre-transplantation sorting would be *in vivo* selection of transduced cells [137,138]. Regrettably, current *in vivo* selection methods use potentially carcinogenic compounds, such as mycophenolate, methotrexate or alkylating agents (i.e., O6-benzylguanine/bis-chloroethylnitrosourea), that offset their use in a disease, such as HIV, that is amenable to an efficient and well-tolerated cART. We want to emphasize that, in our gene-engineering efforts, we aimed for single lentiviral copy integration. The two recent phase I clinical trials used gene engineering protocols that resulted in vector copy numbers of ranging from 2-4 per genome of bone marrow cells prior to transplantation without documenting any adverse events over an observation period of >20 months [132,133]. Thus, ensuring CD34+ transduction might present another alternative for increasing the number of gene engineered CD34+ cells. These protocols appear not to affect the long-term engraftment negatively in these phase I clinical trials.

While the data on a critical threshold in our set of mice are quite evident in the hu mouse model, we do not know what the threshold would need to be in humans. HIV certainly by killing untransduced cells via its cytopathic effects will promote the expansion of HIV resistant cells. To what extent the HIV-resistant cells will foster an efficient HIV-specific immune response and thereby constrain HIV remains unknown.

White blood cell counts from HIV-infected mice generated with FACS sorted R5 KD cells showed an expansion of central memory CD4+ and CD8+ T-cells, while all other groups showed a progressive loss of these CD4+ memory T-cells and no change in CD8+ T-cells. This pattern was also evident when looking at the splenocytes. These memory CD8+ T-cells might have contributed to the control of HIV. There was a decrease in the frequency of naive CD4+ and

CD8+ T-cells in the peripheral blood in both FACS-sorted negative and FACS-sorted R5 KD mice, whereas in the spleen, the naive CD4+ and CD8+ T-cells in the FACS-sorted R5 KD mice tended to be higher (data not shown). The expansion of central memory T-cells is reminiscent of the immune reconstitution seen in HIV-infected patients on ART [139].

HIV is known for its high mutational rate. In this respect, we observed one mouse (#954) with an escape mutation. Despite high levels (day 0) of engraftment of CCR5 knock-down cells, this mouse had a high viral load and a complete loss of circulating $CD4^+$ T-cells. Population sequencing of the V3 loop indicated a likely shift to X4 tropism which might explain the uncontrolled infection. The mutations were detectable in the blood only at relatively late time points. This might be due to an initial compartmentalized replication of the X4-tropic strains in the thymus. We previously showed that X4-HIV NL4-3 severely depleted the thymus, whereas YU-2 only had minor effects [53]. However, we do not know whether the potential emergence was due to the CCR5 knock-down or was just a coincidence. Indeed, emergence of CXCR4-tropic strains may occur without any immune or drug pressure in hu mice infected with CCR5-tropic strains [140]. In any case, CCR5 knock-down should be done in concert with another strategy to constrain HIV (i.e., including another anti-HIV moiety, combining with efficient antiretrovirals, or boosting the immune response in parallel to transplantation). Indeed, the solidness of successful gene engineering by the expression of more than one antiviral moiety may prevent HIV evolution [141]. Gene engineering could be combined with conventional ART: combining two treatment modalities was efficient as induction therapy [142] or with anti-PD1 antibodies that decrease viral load and increase the level of CD4+ T-cells in HIV-infected mice [143]. In any case, gene engineering efforts cannot promote more virulent HIV strains, neither for the individual patient nor for the general population.

In summary, our results provide the first preclinical proof of concept that transplantation of miRNA CCR5 knock-down $CD34^+$ cells can lead to long-term control of HIV viremia. Translation of our results to the clinical setting is relatively straightforward, but will require the

implementation of existing strategies for pre/post-transplantation selection compatible with human use. At this point, our strategy demonstrates long-term viral control, but not yet a cure. However, while a cure remains the ultimate goal, long-term viral control independent of antiretrovirals is a relevant intermediate step, worth translating to the clinical setting. We believe that a definitive cure of HIV might indeed come from a combination of different approaches such as CCR5 knock-down combined with drug therapy or vaccination and a second gene therapy target.

MATERIALS AND METHODS

Ethics statement

The procurement and use of CD34+ cells from human cord blood was approved by the Cantonal Ethical Committee of Zurich (EK-1103). All adult subjects provided written informed consent. Animal care and experimental protocols were in accordance with the "Swiss Ethical Principles and Guidelines for Experiments on Animals," and approved by the Veterinary office of the Canton of Zurich, permit 26/2011. Manipulations of mice were in accordance with the regulations of the Veterinary office of the Canton of Zurich. (http://www.veta.zh.ch/internet/gesundheitsdirektion/veta/de/home.html).

Lentiviral vector production and titration

Lentiviral vector stocks were generated using transient transfection of HEK 293T/17 cells with the self-inactivating vector, pCLX-R4-DEST-R2 encoding the microRNA to CCR5 [129], the psPAX2 plasmid encoding gag/pol, and the pCAG-VSVG envelope plasmid, as described [104]. Lentivector titration was performed using transduction of HT-1080 cells, followed by quantification of GFP-positive (or mCherry positive) cells 5 days after infection by flow cytometry as described [104].

Generation of humanized mice

NOD.scid.IL2R −/− (NSG) mice were bred and maintained in individual ventilated cages and were fed autoclaved food and water. Mice with a human immune system (humanized (hu) mice) were generated as described [118]. Briefly, newborn (<5 days old) NSG mice received sub-lethal (1Gy) total body irradiation with a Cs source, and then received 2× 10^5 transduced or untransduced $CD34^+$ human HSPCs with a 50µl Hamilton syringe via the intrahepatic (i.h.) route. For the FACS-sorted R5 knock-down animals, $CD34^+$ cells were sorted post-transduction into green-fluorescent protein (GFP) positive and -negative fractions, and then, 2x10^5 $CD34^+$ GFP-positive or GFP-negative cells were injected i.h. into respective cohorts. All manipulations

of hu mice were performed under laminar flow. Cell suspensions of the hu mouse organs were prepared in RPMI1640 medium supplemented with 2% fetal calf serum.

HIV virus stock and infection of mice

Viral stocks were obtained by polyethylenimine (PEI)-mediated transfection (Polysciences) of 293T cells with either pYU-2 (R5 tropic) or JRCSF (R5 tropic) (provided through the NIH AIDS Research and Reference Reagent Program). At 48 hours after transfection, virus was harvested, filtered (0.45 μm), and frozen at -80°C until use. Viral titers were determined as described [105]. Briefly, TCID50 (tissue culture infectious dose 50%) was determined by infecting human CD8+ T-cell-depleted peripheral blood mononuclear cells (PBMCs) from three donors that were stimulated by adding IL-2, phytohaemagglutinin (PHA) and anti-CD3 beads (Dynal 11131D, Life Technologies). Mice were infected intraperitoneally (i.p.) with either HIV YU-2 or JRCSF at $2x10^5$ TCID50 per mouse. HIV RNA plasma levels were measured by RT-PCR (AmpliPrep/COBAS TaqMan HIV-1 Test, Roche) at various times after infection.

Flow cytometry

The cells in whole blood were counted in a Beckman Cell Counter. Cell suspensions were labelled with anti-human monoclonal antibodies (mAb) targeting the following cell-surface markers: CD45-PerCP, CD3 ACP, CD4-Pe Cy7, CD8-BVa, CCR5-PE, CD34-APC, CD45RA-APC, and CCR7-PE (all from BD Biosciences or Biolegend). Washing and reagent dilutions were done with FACS buffer (PBS containing 2% fetal calf serum and 0.05% sodium azide. All acquisitions were performed on a Cyan ADP (Beckman Coulter) flow cytometer. Data were analyzed with FlowJo software (Ashland, OR). Cellular debris and dead cells were excluded by their light-scattering characteristics. Transduced CD34+ cells were sorted according to intrinsic GFP expression as measured by a BD FACSARIA™ III cell sorter.

HIV challenge *ex vivo*

Spleens of five hu mice transplanted with HSPCs gene engineered with the microRNA to CCR5 were dissociated through a nylon mesh, and red blood cells were lysed with ACK buffer for 3 minutes (LONZA). Cells were sorted (≥99% pure) with an ARIA sorter (BD Bioscience) into GFP-positive and -negative cells, and were subsequently activated for 24 hours with PHA (4 mg/ml) in RPMI culture medium supplemented with IL-2 (100 U/ml) and 10% fetal calf serum. Thereafter cells were infected with a TCID of 3.3 $x10^5$/ml of YU-2 for 6 hours and washed three times. The supernatant of the last wash was used as the base line p24 antigen level measured by an in house ELISA [106]. Virus spread was then monitored at day 1, 4, 6, 8 and 10 post-infection.

Analysis of HIV envelope sequences

Nucleic Acid Extraction: For viral envelope sequencing, total nucleic acid was extracted from 60 µL of mouse plasma on an EasyMag extractor (BioMerieux, Switzerland) according to the manufacturer's instructions. The elution volume was 50 µL.

Reverse Transcription and PCR: For cDNA synthesis, 9 µL of extracted nucleic acid was reverse-transcribed in a total reaction volume of 20 µL at 42°C for 30 min. using a shorted, sequence-specific primer MSR5 [144] and the PrimeScript One Step RT-PCR kit (Takara Bio Europe/SAS, France). After heat inactivation at 96°C for 5 min. amplification primers were added to the reaction mixture, and DNA corresponding to positions 5956 to 8535 in isolate HXB2 (Accession K03455, M38432) was amplified for 20 cycles. Nested PCR in a total reaction volume of 40 µL with Phusion Hot Start II DNA polymerase (Thermo Scientific, Switzerland) was carried out to amplify, respectively, gp120 (positions 6817 to 7812) and gp41 (positions 7789 to 8382).

DNA Sequencing: Before sequencing, amplified DNA was treated with Illustra ExoStar 1-step reagent (Fisher Scientific, Switzerland). For cycle sequencing, the BigDye Terminator v3.1 Cycle

Sequencing Kit (LifeTechnologies, Switzerland), and specific sequencing primers were used. Twenty-five cycles of heat denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec, and synthesis 60°C for 4 min were carried out on a 2720 Thermal Cycler (Life Technologies, Switzerland). Samples were further processed by ethanol precipitation, followed by capillary electrophoresis on a 3130xl Genetic Analyzer (Applied Biosystems, Switzerland). The sequences were assembled and edited with SeqMan Pro from the Lasergene 11 package (DNAStar).

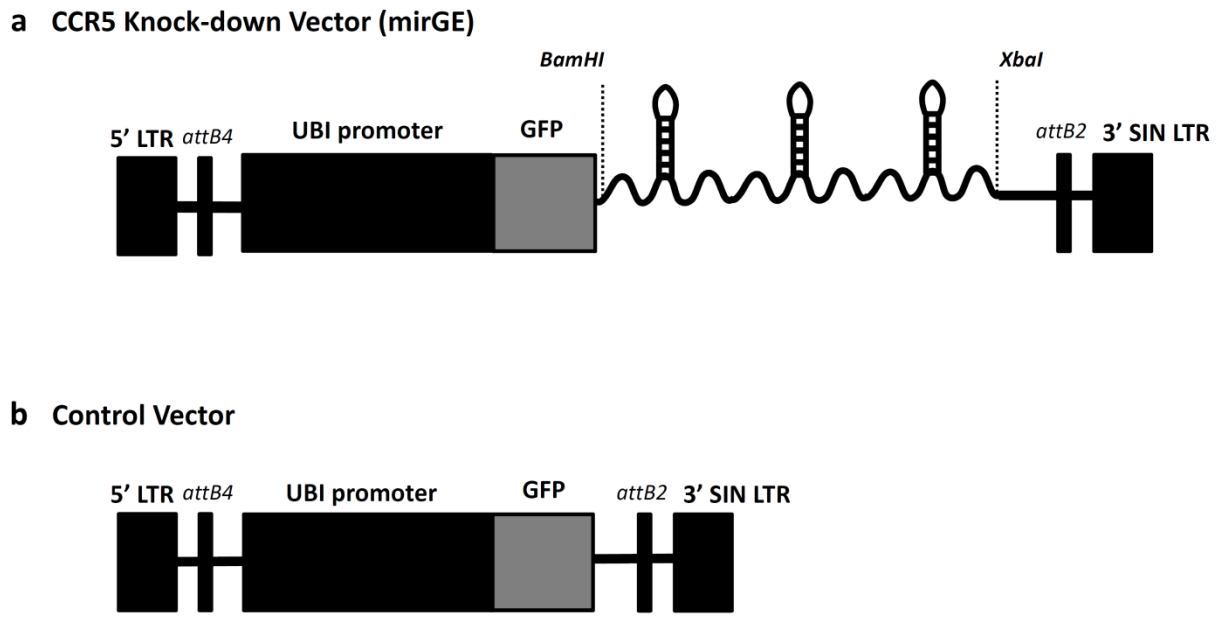
Statistical analyses

The statistical analyses were performed using GraphPad Prism 5.04 (GraphPad Software). Data were subjected to either unpaired t-tests or paired t-tests, as indicated in the figure legends. The *P* values obtained were considered significant when $*P < 0.05$. The statistical outlier analysis was performed using the GraphPad Outlier calculator with an alpha of 0.01 (http://graphpad.com/quickcalcs/Grubbs1.cfm).

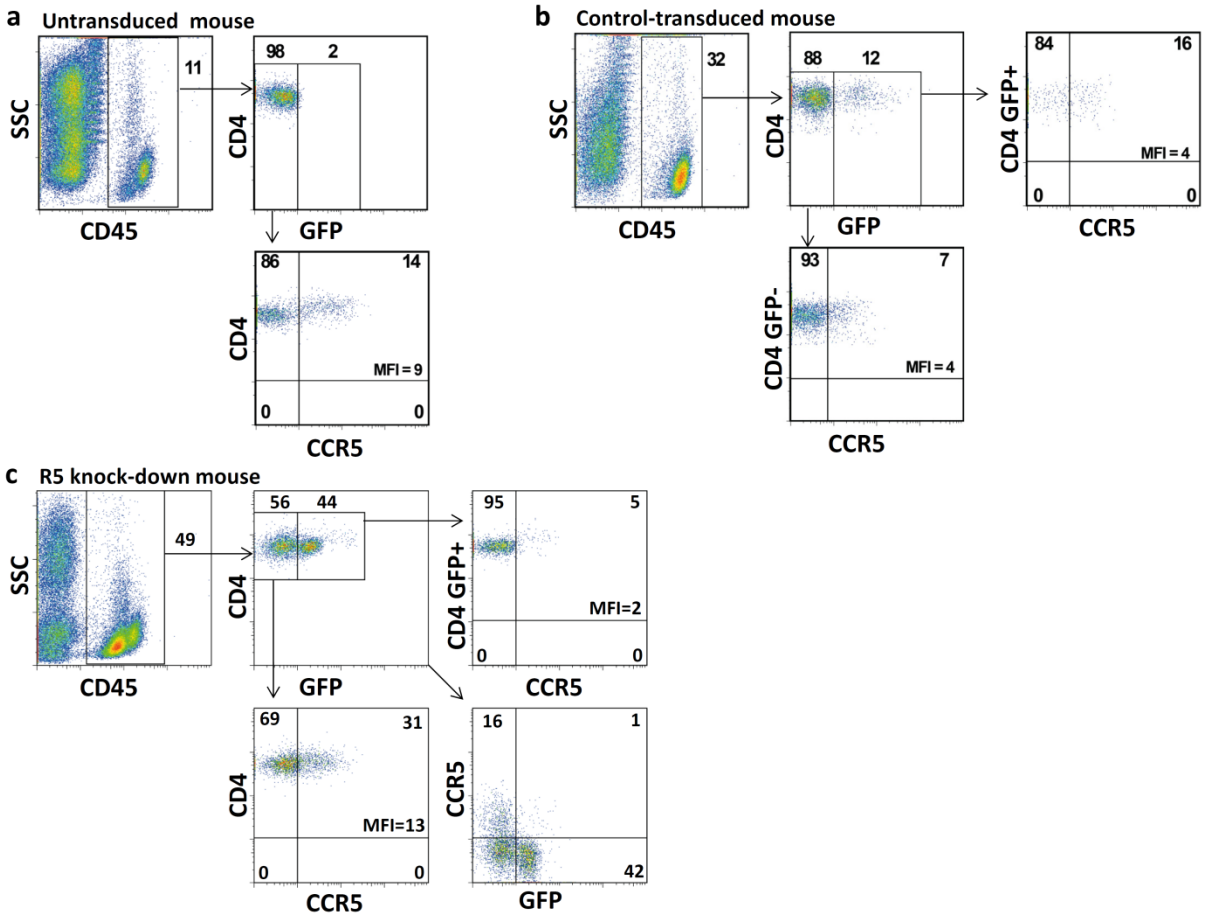
ACKNOWLEDGEMENTS

We thank Astrid Koster, Xenia Rüfenacht and Fatbardha Dautaj (Clinic of Immunology, University Hospital Zurich, Häldeliweg 4, 8044 Zurich) for their work in obtaining the HIV viral loads of the mice. We thank Jürg Böni, Caroline Käser, and Cyril Shah (Institute of Medical Virology University of Zurich, Winterthurerstrasse 190, 8057 Zurich) for performing the sequencing of the HIV genome and analysing the data. This work was supported by the clinical research focus program “Human Hemato-Lymphatic Diseases” of the University of Zürich and the Swiss National Science Foundation, a University of Zurich Candoc grant, the South African National Research Foundation, the Medical Research Council of South Africa and by the Wolfermann Nägeli Stiftung.

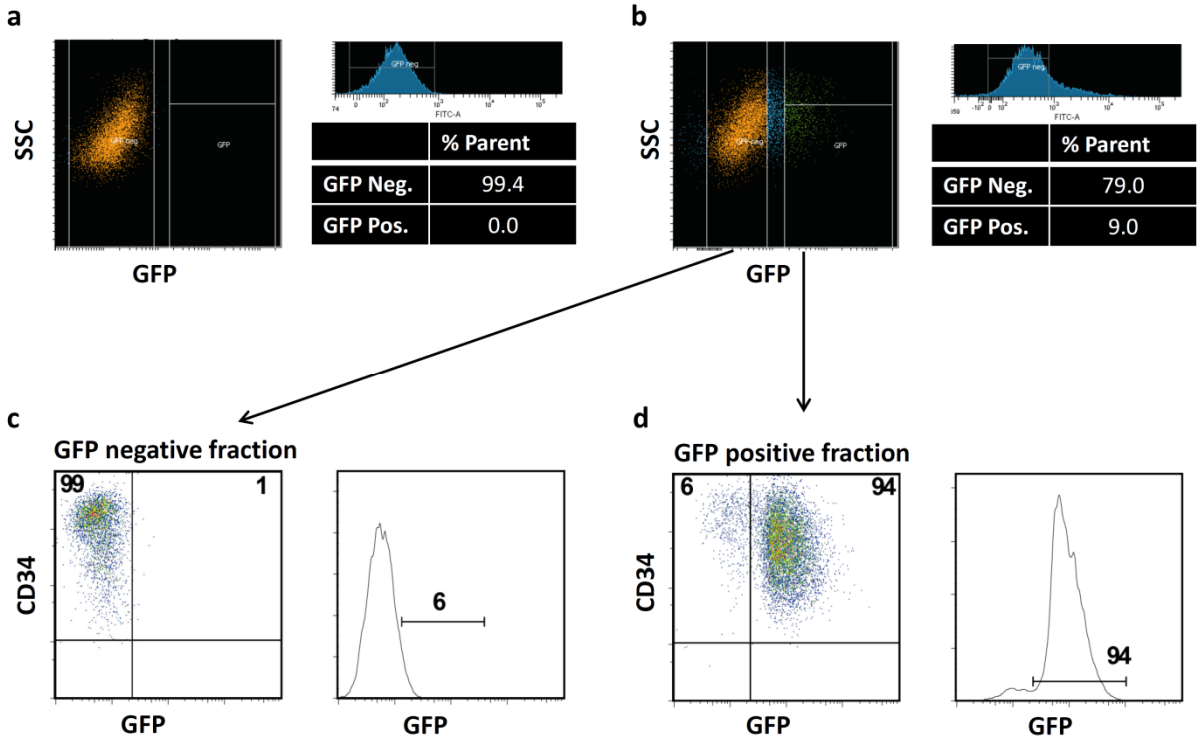
SUPPLEMENTARY MATERIAL



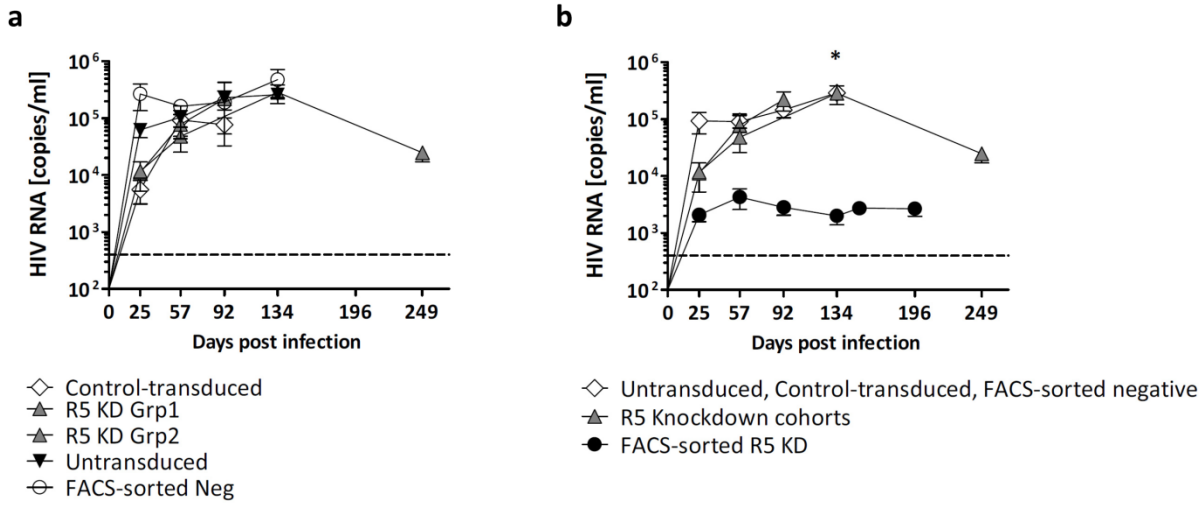
Supplementary Figure 1. Schematic representation of the two self-inactivating (SIN) lentivector constructs used in this study. (a) GFP-CCR5 knock-down lentivector in which the human UBI promoter drives expression of GFP, followed by a triple CCR5-targeting miRNA cassette. (b) Control lentivector, human UBI promoter driving expression of GFP.



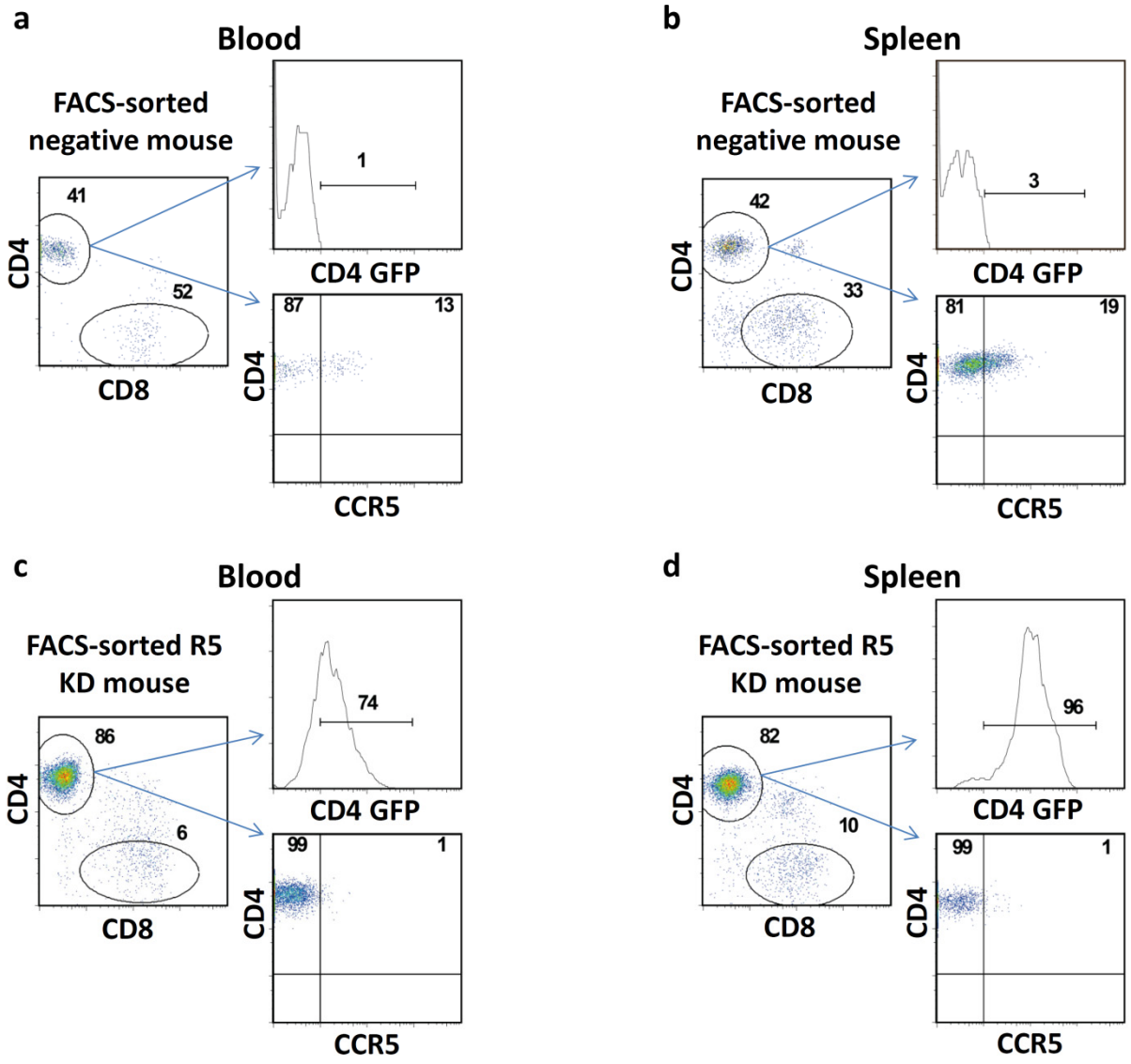
Supplementary Figure 2. CCR5 expression was not detected on GFP-positive CD4⁺ T-cells in blood of R5 knock-down mice. FACS plots showing the percentage human engraftment as quantified by the percentage of CD45⁺cells, as well as percentages of CD4⁺ T-cells and CCR5-positive CD4⁺ T-cells in a non-infected untransduced mouse (a), control (b) and R5 knock-down mouse (c). MFI of CCR5 on CD4⁺ T-cells is indicated.



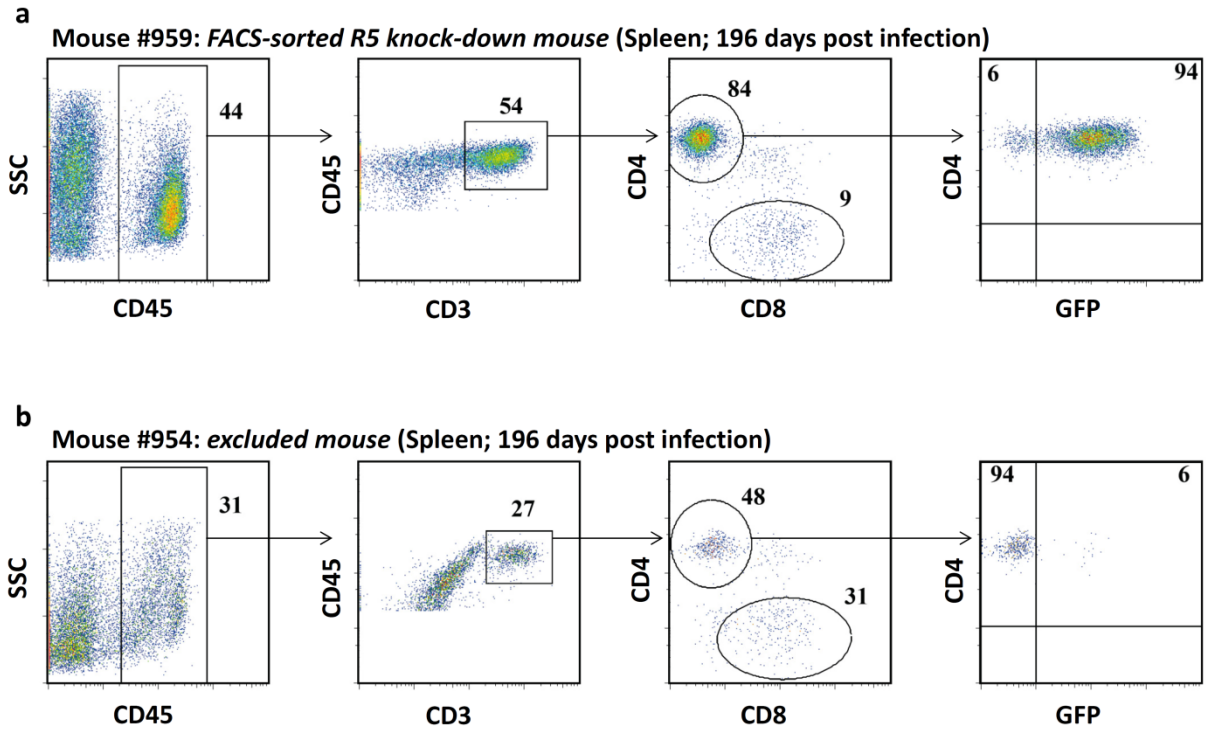
Supplementary Figure 3. Obtaining pure populations of GFP-positive CD34$^+$ progenitors transduced with the CCR5 knock-down lentivector. (a) The untransduced fraction after 3 days of culture. (b) Cells from the same donor as in (a) 3 days post-transduction. The gating for sorting of the GFP-negative and positive populations is show in (b); we sorted only for the GFP high population (9% of the total live population in green) to ensure high purity and high transduction. (c) GFP-negative fraction post-sorting of the cells sorted in (b). (d) The GFP-positive fraction post-sorting of the cells in (b). The cells in the GFP-negative fraction showed a very high purity of 99%; the cells in the GFP-positive fraction were also very pure with 94% of purity.



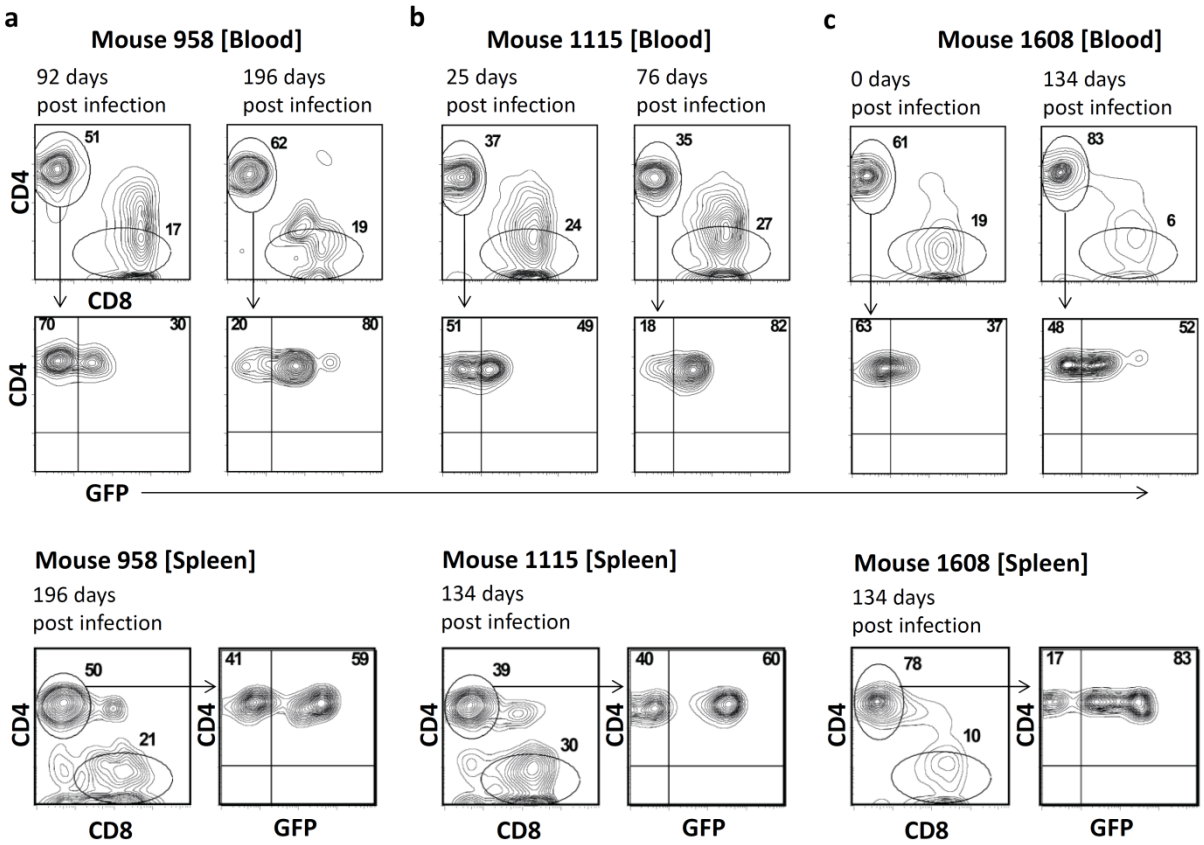
Supplementary Figure 4. Grouped viral loads of various cohorts. (a) control-transduced n=12 (YU-2), R5 knock-down group 1 n=6 (YU-2) and group 2 n=5 (YU-2), untransduced n=11 (YU-2) and FACS-sorted negative n=15 (YU-2 n=9 and JRCSF n=6). (b) The viral loads of the control-transduced, untransduced and FACS-sorted negative mice combined to form the combined control group as well as the two R5 knock-down cohorts compared to the FACS-sorted R5 knock-down mice n=5 (YU-2). Mean ± sem.; **P* = 0.019 (day 134). *P* values determined by two-tailed unpaired *t*-test.



Supplementary Figure 5. CCR5 knock-down on $CD4^+$ T-cells in peripheral blood and spleen of FACS-sorted R5 knock-down mice. FACS plots of peripheral blood and spleen showing, percentage engraftment ($CD45^+$) and $CD4^+$ T-cells, $CD8^+$, CCR5-positive $CD4^+$ T-cells of a FACS-sorted negative mouse (a, b) and of a FACS-sorted R5 knock-down mouse (c, d).



Supplementary Figure 6. Analysis of cell subsets in the spleen of FACS-sorted R5 knock-down mice. (a) Mouse #959 maintained over 90% GFP-positive $CD4^+$ T-cells in the spleen resulting in long term control of viremia. (b) Mouse #954 had very low frequency of gene engineered $CD4^+$ T-cells in the spleen. $CD3^+$ T-cells are of total human $CD45^+$ cells.



Supplementary Figure 7. Detailed analysis of various FACS-sorted R5 knock-down mice. (a) #958(YU-2), (b) #1115(JRCSF) and (c) #1608(JRCSF). FACS plots show percentage $CD45^+$ (spleen only), $CD4^+$ T-cells, $CD8^+$ T-cells and GFP-positive $CD4^+$ T-cells (blood and spleen).

VARIA

In parallel I have published, contributed to and in certain cases initiated various other publications, reviews or projects:

i) Original article *(In review)*

Discovery and characterization of an endogenous CXCR4 antagonist

The author list contains over 40 authors, the affiliations is one page, this is not indicated here.*

This original article is in review. I am a contributing author and conducted various *in vivo* humanized mouse experiments which involved the oral inoculation of HIV viruses to address certain of the reviewers' comments.

Abstract

Endogenous factors controlling HIV-1 replication and transmission 1 are poorly defined. To discover such factors, we screened a blood-derived peptide library and identified a 16-residue fragment (ALB408-423) of human serum albumin (HSA) as a specific inhibitor of CXCR4 (X4)-tropic HIV-1 strains. We found that ALB408-423 is an endogenous antagonist of X4 that inhibits cancer cell migration, mobilizes hematopoietic stem cells and suppresses inflammatory responses in mice. ALB408-423 is highly conserved in mammals and generated by limited proteolysis of HSA by pepsin and cathepsins D and E. ALB408-423 is present in acidified plasma, breast milk, gastric fluid of newborns, vaginal secretions, as well as in oral, vaginal and rectal tissues suggesting that it may represent the long-sought gatekeeper of X4 HIV-1 transmission. Our study identified an endogenous X4 antagonist and demonstrates that

proteolytic cleavage of the highly abundant HSA precursor generates an effective HIV-1 inhibitor and anti-inflammatory agent.

ii) Original article *(In review)*

T Cell Engaging Bispecific Antibody Constructs Targeting HIV-1 Env-expressing Cells Show Significant Antiviral Efficacy

Johannes Brozy*, Erika Schlaepfer‡, Mary-Aude Rochat‡, **Renier Myburgh‡**, Tobias Raum*, Peter Kufer*, Patrick A. Baeuerle*, Markus Muenz*, #, Roberto F. Speck‡, #

# M.M. and RF.S. contributed equally to this work

*** Amgen Research Munich GmbH, Staffelseestr. 2-4, 81477 Munich, Germany, ‡ Division of Infectious Diseases and Hospital Epidemiology, University Hospital of Zurich, Zurich, Switzerland**

This original article has been submitted, I am a contributing author. My contribution was to assist and conduct some of the *in vivo* humanized mouse experiments that were conducted at the University of Zurich.

Abstract

Today's gold standard in HIV therapy is the combined antiretroviral therapy (cART). It requires strict adherence by patients and life-long medication, which can lower the viral load below detection limits and prevent the onset of AIDS, but cannot cure patients. The bispecific T cell engaging (BiTE®) antibody technology has demonstrated long-term relapse-free outcomes in clinical studies with relapsed and refractory acute lymphocytic leukemia patients. We here explored this technology for the treatment of infectious diseases as a potential means to cure patients by redirecting T cells to effectively lyse virus-infected cells. BiTE antibody constructs were generated that target the HIV-1 envelope protein (env) by using variable domains of antibodies B12 and VRC01 or by the first two extracellular domains (1+2) of human CD4. All BiTE antibody constructs showed single-digit nM affinities for HIV env and human CD3e as well as engagement of unstimulated and pre-stimulated T cells for *in vitro* lysis of HIV env-

transfected CHO cells at pM concentrations. Furthermore, anti-HIV-1 env BiTE antibody constructs completely inhibited HIV-1 replication in cultured PBMC and monocyte-derived macrophages in the presence of autologous $CD8^+$ T cells at nM concentrations. Antiviral activity was also observed in NSG mice engrafted with HIV^+ human PBMC by virtue of significantly lowering HIV RNA copies in blood. HIV env-specific BiTE antibody constructs warrant further development as novel antiviral therapy with curative potential.

iii) Original article *(In preparation)*

Studying HIV-triggered EBV-associated lymphoproliferative diseases in a humanized mouse model

Donal McHugh* [a], **Renier Myburgh*** [b], Nicola Caduff [a], Bithi Chatterjee [a], Roberto F. Speck [+b], Christian Münz [+a]

*These authors contributed equally, co-first authors
[+]These authors are co-senior author

This is a project that I initiated with my good friend Donal McHugh at the Institute of Experimental Immunology, Viral Immunobiology also at the University of Zurich. We decided combine our expertise and go for this dual infection (HIV/EBV) model in humanized mice.

Abstract

The ubiquitous gamma herpes virus Epstein-Barr Virus (EBV) latently infects the vast majority of the human adult population. Despite EBV's strong B cell transforming capacity, only few infected individuals develop EBV-associated lymphomas, because of continuous restriction by T cell mediated immune responses. In HIV infected individuals, the drastic loss of $CD4^+$ T cells can therefore lead to EBV-associated Non-Hodgkin lymphomas (NHL). It has been suggested that the HIV induced loss of EBV specific $CD4^+$ T cells results in an exhausted $CD8^+$ T cell population that can no longer control EBV mediated lymphoproliferation resulting in a progression toward NHLs, a hypothesis not yet tested experimentally. Furthermore EBV is highly associated with the development of central nervous system lymphoma (CNSL) in patients with AIDS, a NHL in the brain rarely encountered outside the context of HIV infection. To investigate aspects of pathogenesis and mechanisms of immune control in a small animal model, mice with human immune system components have been successfully used to study HIV and EBV infection in isolation. Recent advances in sophisticated humanized mouse models

have enabled researchers to closely mimic the biology of viruses such as EBV or HIV in vivo. EBV and HIV co-infection is usually found in patients since the vast majority of healthy adults are already latently infected with EBV prior to HIV infection. For this reason we propose to establish a stable in vivo model of dual infection for HIV-1 and EBV. With this model we aim to determine whether loss of EBV immune control via HIV mediated immune suppression leads to the enhanced emergence of lymphomatous tumors. Furthermore we intend to characterize the resulting EBV lymphoproliferative disease with regard to clonality, viral gene expression and spread to different anatomical sites including the CNS. Finally, we aim to characterize the mechanism by which HIV abrogates EBV immune control. The study of EBV and HIV co-infection in humanized mice is a novel concept, which will pool the expertise of two laboratories already independently experienced in EBV and HIV infection in humanized mice (Münz and Speck, respectively). It will be invaluable to investigate an in vivo model in which HIV may compromise EBV specific immune control resulting in EBV associated lymphoproliferative disease. Establishment of such a model with the capacity to recapitulate what is seen in HIV infected patients may pave the way for novel treatments against EBV associated malignancies in HIV infected individuals.

Preliminary results

Co-infection of huNSG mice results in rapid weight-loss, increased EBV viral loads as well as increased frequency of tumors. In a first pilot study, 24 huNSG mice were generated by postnatal irradiation and injection of $CD34^+$ hematopoietic progenitor cells derived from one human fetal liver donor. The animals were checked 4 months later for human leukocyte engraftment in the peripheral blood by flow cytometry and split into four experimental groups each with a similar mean frequency of lymphocyte subtypes: double mock (n=6); HIV (n=5); EBV (n= 7); EBV/HIV (n=6), animals were infected and blood/organs collected according to the timeline (Fig. 1a). We observed a dramatic decline in general condition of some mice in the EBV/HIV infected group from day 21-29 post infection. The sudden and significant weight loss

was striking in the co-infected mice and necessitated termination of the experiment 4 weeks post EBV infection (Fig. 1b). This weight loss was unexpected at this early time point considering the relatively low dose of EBV (10e4 RGU). Upon sacrifice of the animals, macroscopically visible tumors (spleen, liver and gut) were more numerous in EBV/HIV group (Fig. 1c) and mice with tumors in the EBV/HIV group often presented with tumors in multiple organs. The weight loss and increased tumor frequency was accompanied by increased EBV DNA load detectable in the blood of the EBV/HIV cohort at days 21 and 29 post infection in some animals (Fig. 1 d-e).

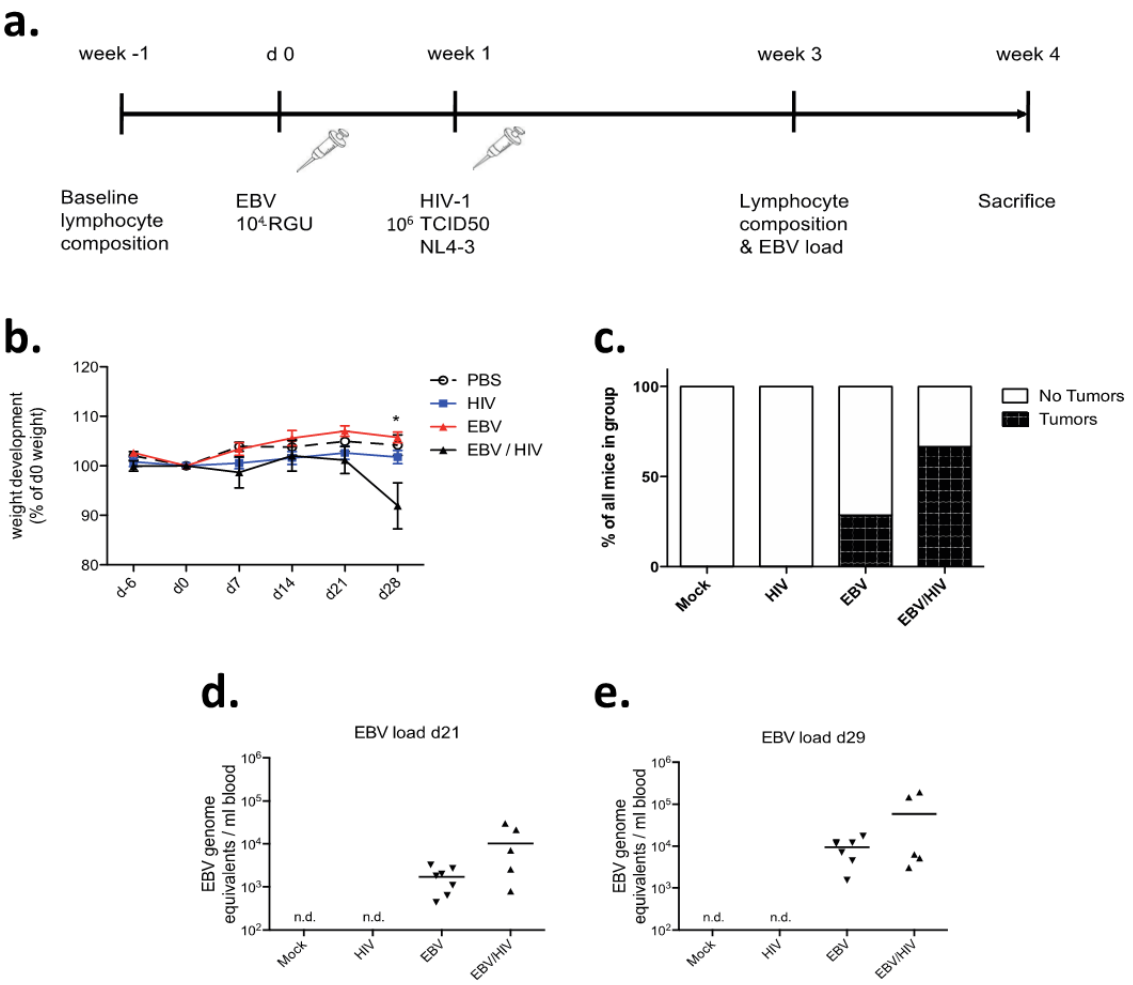


Figure 1. Co-infection of huNSG mice with HIV-1 and EBV leads to weight-loss and increased EBV viral loads in a subset of mice. a) Time line of the experimental set up, mice were infected with 10^4 RGU of EBV or mock infected at day 0. One week later they were infected with HIV-1 or mock infected. The numbers of animals in experimental groups: double mock, n=6; HIV, n=5; EBV, n=7; EBV/HIV, n=6. **b)** Weight development was monitored weekly. Mean relative weight in individual groups is plotted. **c)** Frequency of macroscopically visible tumors upon sacrifice. **d), e)** EBV DNA load in the blood was measured by qPCR.

EBV/HIV infection leads to a loss of CD4+ T cells, expansion of CD8+ T cells and results in a reduced EBV specific IFN-gamma response as compared to EBV single infected mice. One week before EBV infection total peripheral blood human cells, T cells, CD4+ and CD8+ T cells were quantified to ensure the groups were as homogenous as possible and indeed only little variation of the different cell types for the different groups was observed (Fig. 2a). All mice had a normal CD4+/CD8+ ratio, i.e. more CD4+ compared to CD8+ T cells at this time point (Fig. 2a).

At termination, the HIV-mono as well as the HIV/EBV coinfected mice had a significant CD4+ T cell loss in the peripheral blood (Fig. 2b). Upon EBV infection irrespective of HIV, we observed a marked expansion of CD8+ T cells. Thus, HIV- and EBV-mono-infected mice as well as the HIV/EBV-co-infected mice are characterized by an unique pattern of CD4+ T cell loss and/or CD8 T-cell expansion. These data from the blood were mirrored by cellular composition of the spleen (Fig. 2c).

In parallel to the *in vivo* infection experiment, autologous B cells from the donor tissue were transformed with EBV *ex vivo*. The resulting LCLs were co-cultured with B cell depleted (CD19 MACS depleted) spleen cells from each mouse from different experimental groups and the amount of IFN-gamma producing cells was measured by ELISPOT assay. We observed that mice infected with EBV had T cells that released IFN-gamma specifically in response to the LCLs. Most importantly, we could show that the splenic T cells derived from mice in the EBV/HIV infected group had a significantly reduced IFN-gamma response to the autologous LCLs compared to those from EBV single infected mice (Fig. 2d). The overall IFN-gamma response in response to the unspecific stimulus PMA/Ionomycin seemed to be slightly lower in HIV or HIV / EBV infected animals (Fig. 2e). We believe that the loss of the EBV specific IFN-gamma response in the EBV/HIV infected mice could be due to CD8+ T cell exhaustion due to the presence and replication of both viruses. T cell exhaustion markers were analyzed in spleen and blood (data not shown) and there seemed to be an increased surface expression of these markers on CD8+ T cells in the dual infected group. This could explain why there was an increased EBV viral load in

the EBV/HIV mice despite infection with a low EBV dose as well as an early onset of terminal weight loss and increased frequency of spleen and liver tumors.

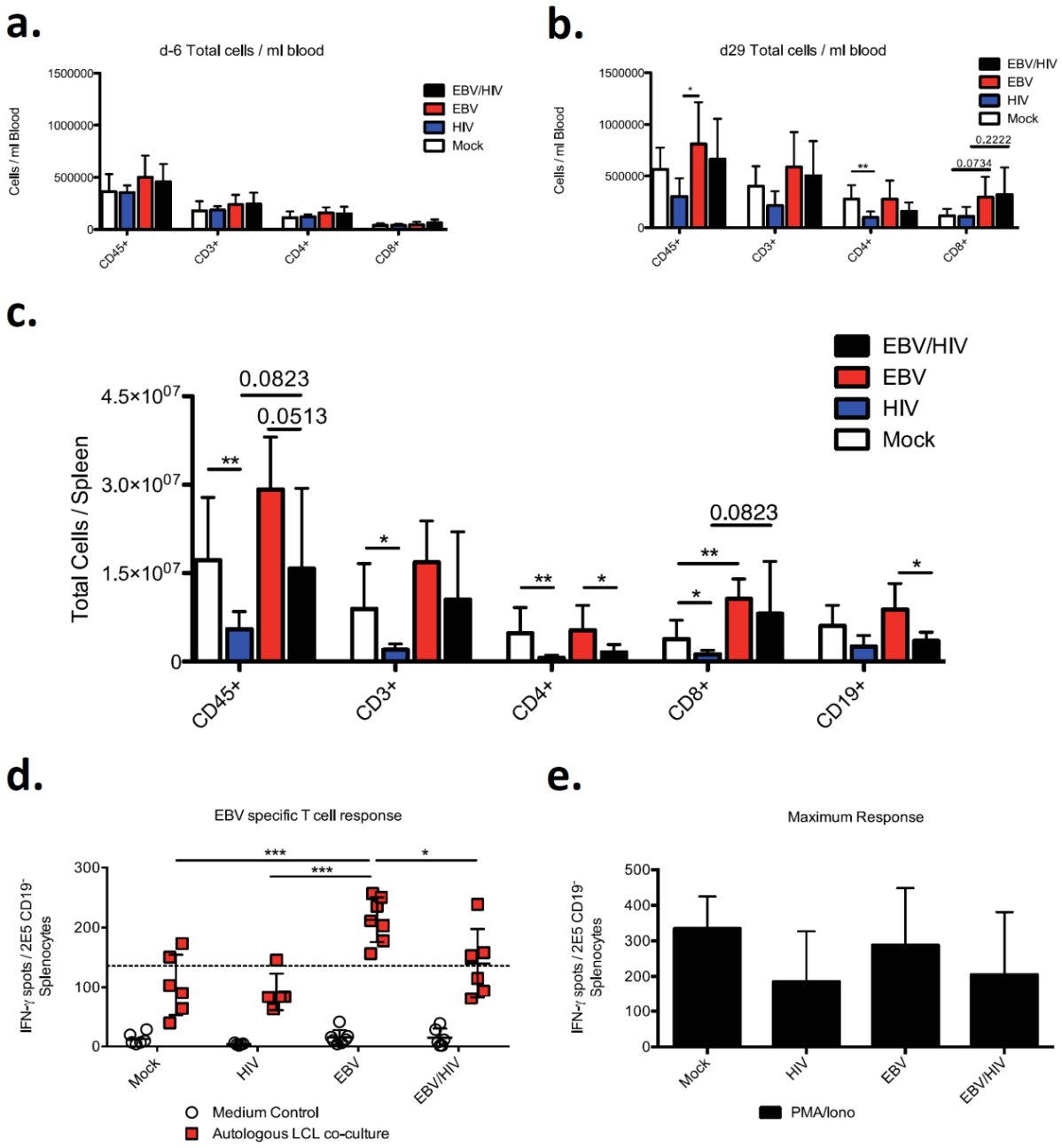


Figure 2. HIV infection leads to reduced CD4+ T cells and loss of EBV specific IFN-gamma response in EBV/HIV co-infected mice. a) Absolute cell composition per ml blood in the respective groups, 6 days before EBV infection as assessed by flow cytometry immune phenotyping. **b)** Absolute cell composition per ml blood on the day of sacrifice. **c)** Total splenic cell composition for the indicated groups is plotted. **d),e)** CD19 MACS depleted Splenocytes were co-cultured with autologous EBV transformed B cells (lymphoblastiod cell line, LCL) and assessed for EBV specific IFN-gamma response via ELISPOT. Plotted values represent means of duplicates for each mouse. **e**) The unspecific response (towards PMA/Ionomycin) is similar and lower in both groups that were infected with HIV compared to the non-HIV infected counterparts.

Significance

Mice with reconstituted human immune system components offer the first small animal models to study pathogens with an exclusively human tropism, as long as they infect cells of the human hematopoietic lineage. The interplay between EBV and HIV, which causes tumors in AIDS patients, can now been studied in these novel *in vivo* models. The proposed project will now address how HIV infection changes EBV infection, which EBV associated tumors form upon HIV co-infection, and how HIV infection compromises EBV specific immune control. These studies will not only characterize the interplay between these two important human pathogens *in vivo*, but also establish a model, which can be explored to test treatments against EBV associated malignancies in HIV infected individuals.

iv) Review article *(In preparation)*

Clinical applications of genetically modified hematopoietic stem cells

Carlo Jackson,[a] **Renier Myburgh [b]**, Karl-Heinz Krause [c], Roberto F. Speck [b] and Michael S. Pepper[a]

[a]Department of Immunology and Institute for Cellular and Molecular Medicine, Faculty of Health Sciences, University of Pretoria, South Africa; [b]Division of Infectious Diseases and Hospital Epidemiology, University Hospital of Zurich, University of Zurich, Switzerland, University of Geneva Hospitals, Geneva, Switzerland; [c](Karl-Heinz).

This is an on topic review which I have contributed to as the second author. This manuscript is in preparation and planned to be submitted before the end of 2014

Abstract

Autologous and allogeneic hematopoietic stem cell (HSC) transplantations have been performed globally in patients with various haematological and other disorders for more than 50 years. Genetic manipulation of HSCs prior to transplantation has been envisaged for genetic and infectious diseases to overcome limitations associated with naive HSCs. However, many obstacles need to be overcome before HSC-mediated gene therapy can be considered for routine therapy. A major concern is that the integration of the genetic elements might cause endogenous gene activation or deactivation that in turn lead to aberrant cell proliferation and tumour formation. Many improvements have been made in vector construction aimed at overcoming this problem, but the optimization of other facets of the gene therapy process could further increase the safety of the treatment. The transduction efficiency of HSCs is much lower

than most cell types and is an obstacle to obtaining adequate numbers of transduced cells capable of engrafting in adult patients. Furthermore, HSCs generally differentiate in culture and lose their ability to engraft, and thus it is not a trivial task to obtain transduced HSCs that have not lost their ability to provide long term engraftment. We provide an overview of several facets involved in HSC gene therapy including the success stories and limitations of clinical trials and the latest techniques that can be used to improve the process.

v) Review article *(Published: Progress in Neurobiology. 2011. Vol. 93. Pg.293-311)*

The chemokine receptor CCR5 in the central nervous system

Silvia Sorce [a], **Renier Myburgh [a,b]**, Karl-Heinz Krause [a,c,*]

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This is a review which I was a contributing author. Various chapters were my responsibility.

Abstract

The expression and the role of the chemokine receptor CCR5 have been mainly studied in the context of HIV infection. However, this protein is also expressed in the brain, where it can be crucial in determining the outcome in response to different insults. CCR5 expression can be deleterious or protective in controlling the progression of certain infections in the CNS, but it is also emerging that it could play a role in non-infectious diseases. In particular, it appears that, in addition to modulating immune responses, CCR5 can influence neuronal survival. Here, we summarize the present knowledge about the expression of CCR5 in the brain and highlight recent findings suggesting its possible involvement in neuroprotective mechanisms.

vi) Review article *(Published: Pharmacology & Therapeutics. 2012. Vol. 133. Pg. 280-290.)*

Cardiovascular Pharmacogenetics

Renier Myburgh [a], Warren E. Hochfeld [b], Tyren M. Dodgen [c], James Ker [d], Michael S. Pepper [a,e,*]

[a] Department of Immunology, Faculty of Health Sciences, University of Pretoria, South Africa, [b] Department of Medical Genetics, School of Clinical Medicine, Cambridge University, United Kingdom, [c] Department of Pharmacology, Faculty of Health Science, University of Pretoria, South Africa, [d] Department of Internal Medicine, Steve Biko Academic Hospital and Faculty of Health Sciences, University of Pretoria, South Africa, [e] Department of Genetic Medicine and Development, Faculty of Medicine, University of Geneva, Switzerland

Since my PhD project is an ongoing collaboration between three Universities (University of Zurich, Geneva and Pretoria in South Africa) I had spent time at all three institutions. Certain institutions encourage the writing of review articles for the thesis that are on different topics than that of the main research focus. For this reason I published this "off topic" review as first author during my time in South Africa and Geneva.

Abstract

Human genetic variation in the form of single nucleotide polymorphisms as well as more complex structural variations such as insertions, deletions and copy number variants, is partially responsible for the clinical variation seen in response to pharmacotherapeutic drugs. This affects the likelihood of experiencing adverse drug reactions and also of achieving therapeutic success. In this paper, we review key studies in cardiovascular pharmacogenetics that reveal genetic variations underlying the outcomes of drug treatment in cardiovascular disease. Examples of genetic associations with drug efficacy and toxicity are described, including the roles of genetic variability in pharmacokinetics (e.g. drug metabolizing enzymes)

and pharmacodynamics (e.g. drug targets). These findings have functional implications that could lead to the development of genetic tests aimed at minimizing drug toxicity and optimizing drug efficacy in cardiovascular medicine.

vii) Review article *(Published: European Journal of Cell Biology. 2013. Vol. 92. Pg. 229-236)*

Adipocyte and adipogenesis

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Abstract

Adipocytes are the main constituent of adipose tissue and are considered to be a corner stone in thehomeostatic control of whole body metabolism. Their primary function is to control energy balance bystoring triacylglycerol in periods of energy excess and mobilizing it during energy deprivation. Besides theclassical function of storing fat, adipocytes secrete numerous lipid and protein factors. Collectively theyare considered constituting a major endocrine organ which has a profound impact on the metabolismof other tissues, the regulation of appetite, insulin sensitivity, immunological responses and vasculardisease.Adipogenesis is the process during which fibroblast like preadipocytes developed into matureadipocytes. Adipogenesis is a well-orchestrated multistep process that requires the sequential activationof numerous transcription factors, including the CCAAT/enhancer-binding protein (C/EBP) gene familyand

peroxisome proliferator activated receptor (PPAR). In order to reach maturity, these cells mustgo through two vital steps: adipocyte determination and adipocyte differentiation. Although many of themolecular details of adipogenesis are still unknown, several factors involved in this processes have beenidentified. Some stimulators include peroxisome proliferator-activated receptor (PPAR), insulin-likegrowth factor I (IGF-l), macrophage colony stimulating factor, fatty acids, prostaglandins and glucocor-ticoids. Inhibitors include glycoproteins, transforming growth factor (TGF), inflammatory cytokinesand growth hormone. Beside these factors, there are others for example age, gender and life style thatmay affect this process in one way or another. An increase in the number and size of adipocytes causeswhite adipose tissue (WAT) to expand and this can lead to obesity. Adipogenesis can lead to centralobesity if it occurs in the abdominal fat depot and peripheral obesity if it occurs in subcutaneous tissue.

viii) Project underway

Development of an *in vitro* and/or *in vivo* selection strategy for positive and negative selection of transduced CD34$^+$ progenitor cells

Working Hypothesis

We hypothesize that gene engineering of hematopoietic progenitor cells (HPC) can give rise to an HIV resistant immune system, this approach applied through autologous manipulation and transplantation of HPCs represents a promising approach for a cure of HIV. Gene engineering will be achieved using lentiviral based self-inactivating (SIN) vectors targeting the HIV co-receptor CCR5 and eventually other viral targets. In addition, we hypothesize that positive selection of gene engineered CD34$^+$ cells is mandatory since the majority of progeny cells are required to be resistant to HIV; if not the unmodified HIV target cells will permit establishment of latent reservoirs and ongoing viral replication resulting in progressive immunodeficiency and potential horizontal or vertical HIV transmission.

Tumor cells are known to get resistant to chemotherapeutic drugs, which is not the case for, however "normal" cells. Therefore, the expression of genes in normal bone marrow cells that increase their tolerance to drugs would allow for efficient negative selection of the untransduced cells [145].

Specific Aims

1. To study strategies for in vitro/vivo selection of gene engineered CD34$^+$ cells

1.1 To investigate the potential to assert positive and negative selection pressures by expression of the human reduced folate carrier (hRFC), and (RRM2) gene in CD34$^+$ cells. When cells are exposed to either Trimetrexate and/or Hydroxyurea as well as Methotrexate a

different selective pressure can be affected. *Please see section viii), the development of this vector is currently under submission for patenting.*

1.2 To develop a selection system based on the dihydrofolate reductase gene (DHFR), where a mutant version of this gene ($DHFR^{fs}$) provides cells over expressing this gene with resistance to Methotrexate and thus allow for positive selection of transduced cells [137].

Introduction and relevance

In collaboration with the laboratory of Prof. Krause (UNIGE) we developed a vector which combines an *in vivo* selection as well as a suicide strategy based on the expression of the human reduced folate carrier (hRFC1) and the ribonucleotide reductase subunit (RRM2) [146,147]. Early gene therapy trials with retroviral based vectors resulted in a substantial numbers of patients developing lymphomas due to the integration of the retroviral based vectors into proto-oncogenic sites. While the risk of such a serious adverse event using lentivirus based vectors is highly unlikely, gene engineering strategies should include a suicide strategy for killing of gene engineered cells which might undergo malignant transformation. Since HIV replication will not be controlled when only a minority of cells are resistant, selection is needed for the enrichment of gene engineered cells pre- or post-transplantation.

1.1 hRFC1 is a transport protein for folate compounds and in particular also for the folate antagonist methotrexate. Thus, increased expression of hRFC1 may promote the uptake of methotrexate and thus the preferential killing (suicide) of transduced cells; untransduced cells will be more resistant. In contrast, trimetrexate, another folate antagonist, is lipophilic, and thus is not dependent upon hRFC1; thus, cells with increased expression of hRFC1 will have an increased transport of folinic acid which will render cells resistant to trimetrexate (selection); untransduced cells will be more vulnerable to this compound. In other terms, hRFC1 gene engineered HPCs and their progeny cells should be highly vulnerable to the effect of methotrexate (suicide) while being resistant to trimetrexate (selection) [146,148].

1.2 Human DHFR is involved in synthesis of purines and thymidylates the basic components used to build DNA. More specifically, DHFR reduces dihydrofolic acid (a vitamin B9 derivative) to tetrahydrofolic acid which is the bases for synthesis of purines and pyrimidines [149]. Methotrexate inhibits DHFR and is able to prevent the proliferation and growth of T cells [150]. A mutant form of the DHFR gene has been described called $DHFR^{fs}$, which has normal enzymatic activity but due to two point mutations (Leu22 to Phe and Phe31 to Ser) methotrexate cannot bind to and inhibit the mutant form of the enzyme [151].

Trimetrexate and methotrexate are both compounds with FDA approval for human use. Trimetrexate was used for the treatment of pneumocystis carinii pneumonia. In a first instance we will examine the *in vitro* selection/killing by transducing HeLa cells or SupT1 cells with the vector encoding hRFC1 which then will be exposed to either methotrexate or trimetrexate. We will be able to verify enrichment or killing of transduced cells by tracking the frequency of GFP positive cells by flow-cytometry when adding trimetrexate or methotrexate since the vector encodes in addition GFP.

Preliminary results

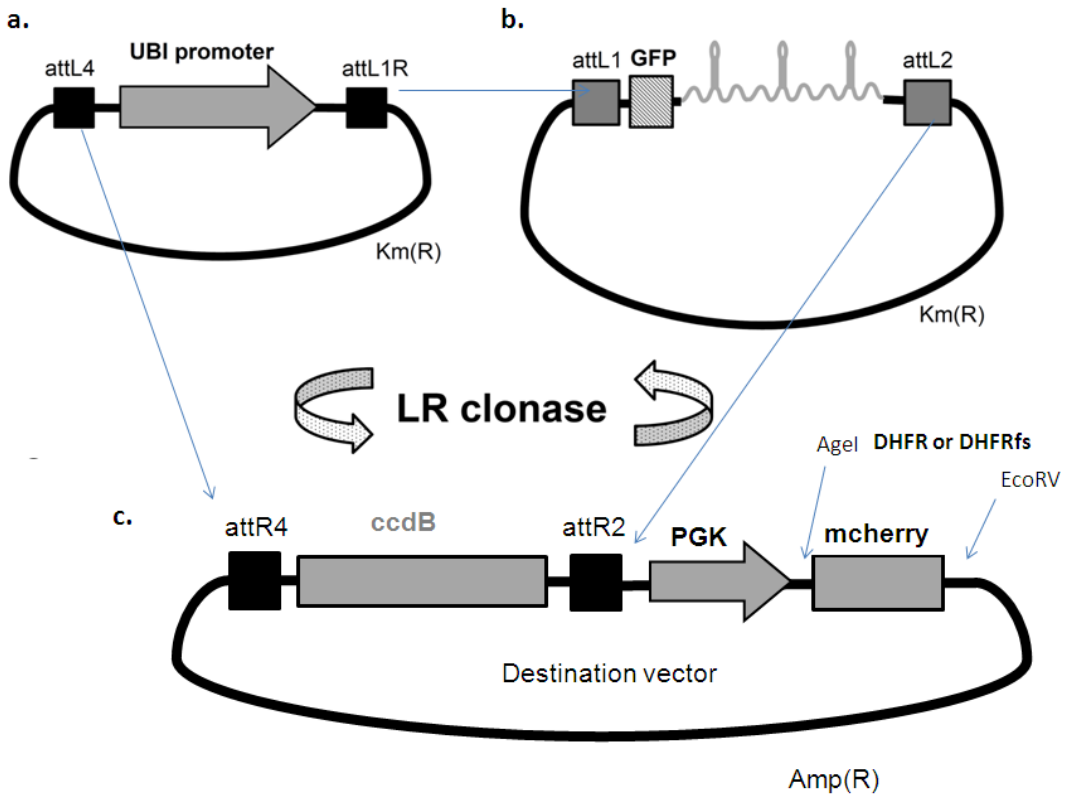

Figure 1. Cloning strategy for the DHFR and DHFR^{fs} selection project (please see section 1.2 above). Recombinational cloning to prepare the DHFR and DHFR^{fs} selection plasmid which would include expression of the GFP reporter gene and the CCR5 targeting triple microRNA cassette. a) pENTR plasmid comtaining the human ubiquitin C promoter (UBI). This plasmid allows for bacterial selection with kanamycin. b) pENTR plasmid containing the GFP reporter gene followed by the CCR5 targeting microRNAs. This plasmid allows for bacterial selection with kanamycin. c) HIV-1 based self-inactivating expression vector which contains the HIV-1 LTRs and other critical cis-acting elements to allow packaging of lentivector particles as well as insertion if transgenes into the target cell genome. This plasmid contains the human PGK promoter with am excisable mCherry gene allowing for insertion of a selection gene using AgeI and EcoRV restriction sites. Once an enzymatic LR recombinational reaction is carried out, the UBI promoter and GFP-CCR5 microRNA sequences will be inserted in place of the ccdB toxic gene. This plasmid allows for bacterial selection with Ampicillin.

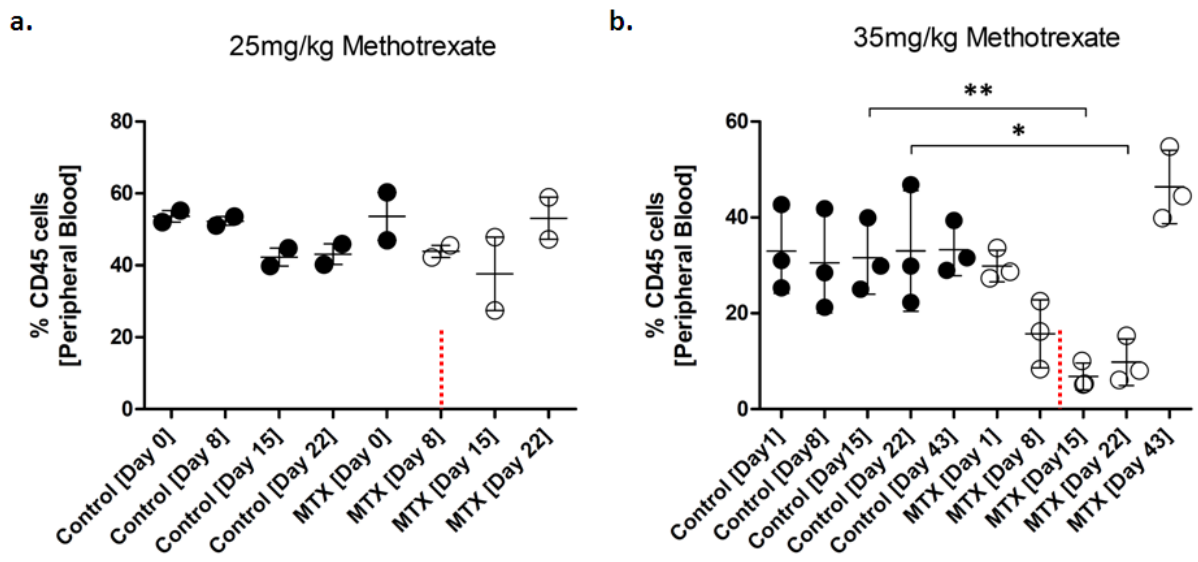

Figure 2: Administration of methotrexate causes a transient ablation of human cells in humanized mice at a dose of 35mg/kg per mouse. a) Control mice (n=2) and methotrexate mice (n=2) were injected with PBS and 25mg/kg methotrexate respectively at days 0, 4 and 8. There was no difference in frequency of human cells ($CD45^+$) in the peripheral blood during the course of the experiment. The red dotted line indicates the last day of drug administration. b) Control mice (n=3) and methotrexate mice (n=3) were injected with PBS and 35mg/kg methotrexate respectively at days 1, 4, 8 and 11. At day 15 there was a further decrease in frequency of human cells ($CD45^+$) compared to day 8. At day 15 and 22 the mice receiving methotrexate had significantly lower frequencies of human cells ($CD45^+$) compared to the PBS mice. 32 days after the last injection, the frequency of human cells had recovered in all mice that received methotrexate. The red dotted line indicates the last day of drug administration.

Outlook

Once preliminary testing is complete the vectors described in this thesis would require optimization of the geometry to produce the final vector which will combine the elements for in vivo selection/suicide and two anti-HIV targets. This will be conducted in collaboration with the laboratories of Prof. Karl-Heinz Krause (UNIGE) and Prof. Michael Pepper (University of Pretoria, SA) to initiate a phase I clinical trial.

ix) Invention disclosures

University of Geneva, UNITEC: New design of micro RNAs (miRNAs) for gene therapy vectors

We have an MTA that the three universities involved in this project (University of Zurich, University of Pretoria and the University of Geneva) all have rights to use these inventions

Inventors: **Renier Myburgh**

Contributors: Karl-Heinz Krause, Patrick Salmon

Design of effective and improved gene therapy vectors enabling markedly improved efficiency of specific gene knockdown. Invention circumvents known weak points in published designs. Vectors enabled by the invention could have therapeutic applications in a range of diseases, including treating haematopoietic stem cells for treatment of malignancies and HIV infection.

University of Geneva, UNITEC: *in vivo* cell selection via gene therapy and combination cytotoxic treatment

We have an MTA that the three universities involved in this project (University of Zurich, University of Pretoria and the University of Geneva) all have rights to use these inventions

Inventors: **Renier Myburgh**, Karl-Heinz Krause, David Suter

Contributors: Michael Pepper, Patrick Salmon

New strategies for *in vivo* selection through the use of gene therapy vectors containing resistance genes against (more than one) cytotoxic compounds. Enabling *in vivo* (positive or negative) selection of transplanted cells concomitantly transformed by disease-specific gene therapy, thus favouring survival of cells with effective therapeutic modification.

DISCUSSION

In this thesis, we endeavoured to develop a gene therapy cure for HIV. We based our strategy on producing CCR5-deficient hematopoietic progenitor cells, which are CCR5 deficient as a result of constitutive expression of highly efficient CCR5 targeting microRNAs (miRNAs).

We had several reasons for using this strategy. First, HAART treatment has improved the outcome for HIV patients in general but has known disadvantages, such as drug toxicity (cardiovascular, kidney, liver disease as well as cancer), the need to adhere to complicated drug regimens, and the emergence of drug-resistant strains of the virus [152-155]. Second, CCR5-deficient individuals are protected against HIV infection yet suffer no detrimental effects without CCR5 [156]. Third, in South Africa, a substantial number of HIV-infected patients also have lymphomas in advanced stages; this included AIDS defining and non-AIDS fining cancers. [157,158]. Chemotherapy alone or in combination with rituximab will not be sufficient to cure these lymphomas, and these patients will still require treatment with ablative chemotherapy and autologous stem cell transplantation. This provides the opportunity to intervene and complement the autologous hematopoietic progenitor cells of these patients with anti-HIV genes able to eliminate CCR5 expression to try and cure HIV in parallel.

The proof of concept that CCR5 deficiency can be used as a therapy for HIV is the case of the "Berlin patient," who received donor CCR5-deficient hematopoietic progenitor cells to treat acute myeloid leukemia. The patient became aviraemic soon after transplantation [43]. This is still, however, a unique case [51], and it does not strongly suggest CCR5 as a viable gene therapy target. This does not mean that other targets for HIV gene therapy should be ignored; indeed many groups have explored the potential of targeting HIV genes, such as Tat, Nef and Rev (only to mention a few), with RNA interference to eliminate expression of these gene products [159-162]. The concern with targeting HIV genes is that a single target of this kind should not be used in

isolation. HIV has a very high replication rate that ultimately results in mutations in the HIV RNA that render the gene therapy useless.

Effective and robust targeting of HIV genes with RNA interference requires the use of several dissimilar RNA interference species that target different genes. Furthermore, the RNA interference genes must have perfect homology to their HIV gene targets: even a single mutation in an HIV gene can prevent targeting by the RNA interference gene [163-165]. Thus, the most robust strategy would be to target a host gene, such as CCR5, as well as a single HIV gene or multiple HIV genes, providing broad enough coverage to prevent HIV escape. Indeed this type of strategy has been studied in humanized mice in which down-regulation of CCR5 with an shRNA was combined with a Tar decoy and an anti-HIV TRIM5α isoform [56]. However, this complicated strategy did not inhibit viral load *in vivo.* Only when splenocytes from these mice were sorted into anti-HIV transduced and non-transduced cells did the authors report that HIV replication was blocked in the anti-HIV transduced fractions [55,56,166].

We opted to start with development of a CCR5-based strategy before developing secondary and/or tertiary targets. It is also important to verify the effectiveness of each single target [54,59-61,167], before testing multiple targets simultaneously [55,56,168-170]. Silencing/knocking-out CCR5 by shRNAs or zinc-finger nucleases has been extensively tested in HIV-permissive humanized mouse models [54-56,59]. Up to this point, miRNAs to knock-down CCR5 have not been tested in humanized mouse models. This is somewhat surprising since shRNAs cause cellular toxicity *in vivo* [65,171]. The toxicity has been linked to shRNAs having to be expressed from highly active pol III promoters, however these promoters are usually necessary for proper expression of shRNAs as they require very precise ends to be correctly processed and he initiation and termination sites of pol III promoters are well defined [172]. Furthermore, shRNAs bypass DROSHA processing in the nucleus and over-saturate exportin5, which affects the processing of naturally occurring miRNAs in the cell [65,171]. On the other hand, miRNAs can be expressed from less active pol II

promoters and, since they follow the full RNA interference pathway (including DROSHA processing), might be less likely to disrupt cellular homeostasis [172]. The rate of transduction must also be considered in terms of causing cellular toxicity. If excessive transduction rates are used (over 50%), the resulting high level of expression due to the presence of multiple copies of the transgene would likely result in cellular toxicity even with miRNAs expressed from pol II promoters [89,172].

Since these gene therapies are aimed at patients, safety is of great concern, and the choice of vector is important. A previous gene therapy trial for (SCID) X-1 disease used moloney murine leukaemia gamma retrovirus (MLV). The resulting activated proto-oncogenes caused T-cell leukemia in the patients [173,174]. The tendency of gamma-retroviruses to integrate their genomes near transcription start sites and Cpg islands was directly linked to the development of leukaemia. A further consideration is level of transduction. High levels of transduction increase the number of transgene copies inserted per cell [89], and a combination of high rates of transduction and the tendency of gamma-retorviruses to integrate in the same regions in close proximity to known proto-oncogenes increases the chance of aberrant effects [89,132,133,173].

With safety in mind, our CCR5-targeting miRNA was cloned into a self-inactivating HIV-1-based lentivector system, and we maintained reasonably low levels of transduction (<30%). In human gene therapy clinical trials for Wiskott Aldrich syndrome and metachromatic leukodystrophy, HIV-1-based lentivectors have been, thus far, safe in terms of the integration profile even when high transduction rates were used [132,133]. The reason for this is lentivectors tend to insert within transcriptional units or gene-rich regions and not close to genes that are associated with causing clonal expansion [132,133]. A further advantage of lentivectors is their ability to transduce quiescent cells. HIV-1 lentivectors can better transduce quiescent, primitive human hematopoietic progenitor cells than MLV-based vectors. Because anti-HIV transduced progeny cells are required throughout life, HIV gene therapy must achieve decent levels transduction of

true hematopoietic progenitors [175]. The ability of HIV-1-based lentivectors to transduce quiescent cells is why lentivectors have such a distinct and much more favourable integration profile than MLV vectors.

We used an HIV-1-based lentivector with a CCR5-targeting miRNA cassette (three hairpins in tandem) and limited transduction rates to approximately 20% to prevent cells from containing more than one copy the transgene. The limited transduction rates make knock-down of CCR5 more difficult since the miRNA needs to be efficient enough to achieve high-level knock-down despite the low transduction rate. The first miRNA we tested, miR-30, resulted in suboptimal knock-down at low transduction rates (<20% knock-down). Since miR-30 was commercially available along with a well-described system for target gene knock-down (GIPZ & TRIPZ shRNAmir lentivector expression systems, Thermo Scientific Open Biosystems; http://www.bmgc.umn.edu/prod/groups/ahc/@pub/@ahc/@bmgc/documents/asset/ptripz_technical_manual.pdf), we were forced to find a way to significantly improve its efficiency. We attempted to redesign the miRNA to improve its processing by endogenous RNA interference enzymes which would result in more targeting strand being released in the cytoplasm to allow for greater knockdown of the target gene. It must be efficient enough to achieve complete knockdown at low transduction rates. We hypothesised that the poor performance of miR-30 was due to an inefficient processing somewhere between Drosha and DICER. Through an in-depth review of the literature, we found that weaker down regulation was provided by miRNAs with shorter lower stem lengths and with upper stems in which the 5' end of the targeting strand was not thermodynamically unstable compared to the 3' end [72]. miR-30 miRNAs have a shorter lower stem and suboptimal targeting strand thermodynamic configuration. We thought this would negatively affect DROSHA processing and hamper release of targeting strand. To address these issues, we modified various structural features of the miRNA that we called (mirGE). The modifications were the following: the lower stem length was increased from 9 to 13 nucleotides, the targeting strand was moved to the 5' side of the miRNA, and the 5' end of the

targeting strand was designed to be more thermodynamically unstable than the 3' end, which should facilitate selection and loading of the targeting strand into RISC rather than the passenger strand. [72,93,176] . We also included a wobble in the middle of the upper stem to prevent abortive processing [72]. These modifications resulted in 2x more CCR5 knock-down than with miR-30 at a similar transduction rate.

While refining our design, we discovered that the thermodynamically unstable 5' end of the targeting strand was not so important for improving knock-down efficiency as described [72,93,176]. To test this, we developed various hairpins targeting GFP with lower stem lengths ranging from 9–17 nucleotides and where either the 5' or 3' of the targeting strands were thermodynamically unstable. These experiments showed that it was rather the increased lower stem length (>11 nucleotides and <17 nucleotides) that increased GFP knockdown since within this lower stem length range, the 5' targeting strand mismatch did not significantly increase GFP knock-down, compared to the same hairpins with an unstable 3' targeting strand. These results were confirmed by sequencing total RNA from cells transduced with the miR-30 or mirGE vectors. We used next-generation sequencing techniques to detect the siRNAs (targeting and passenger strands) released by DROSHA and DICER processing. The increased level of knock-down capacity of our mirGE could be attributed to the increased lower stem length, which resulted in very accurate DROSHA processing (0.027% incorrect cuts), compared to miR-30, with a shorter lower stem in which DROSHA processing was very inaccurate (21% incorrect cuts). For mirGE, there was very efficient release of the 5' end of the targeting strand. For miR-30, since the targeting strand was carried on the 3' end of the hairpin, DICER was responsible for releasing of its targeting strand 5' end and for both mirGE and miR-30 DICER was relatively imprecise in its cleavage. This resulted in 10x more targeting strand available for RISC coming from mirGE compared to miR-30.

The mirGE design proved successful during *in vitro* testing where with a triple miRNA expressed as a cassette we could achieve >90% CCR5 knockdown at low transduction rates in HeLa cells. We then initiated *in vivo* testing. We used the NSG humanized mouse model, where mice were produced with transduced human umbilical cord blood derived $CD34^+$ progenitor cells. In the first set of experiments, we transduced $CD34^+$ cells at <30% with the CCR5 knock-down vector and transplanted the total $CD34^+$ cell population into the mice. In these animals, less than 30% of the progeny cells were transduced *in vivo,* and no viral load inhibition was achieved. The major limiting factor was the low frequency of anti-HIV transduced cells. This was the same limiting factor that hindered various previous attempts at inhibiting HIV viral load in humanized mice with gene therapy [55,56,59,177]. Shimizu et al; transplanted mice in which 45–50.8% of $CD34^+$ cells were anti-HIV transduced [59]. Ringpis et al; transduced the $CD34^+$ cells in a range of 63–79%, however, mice were transplanted with a 50:50 mixture of transduced and untransduced cells [55]. Walker et al; did not indicate the initial transduction rate of their $CD34^+$ cells, but mice transplanted with the anti-HIV transduced $CD34^+$ cells had 17.5% of the total human cells and 55% of $CD4^+$ T cells transduced *in vivo* [56]. Centlivre et al; transplanted mice with a $CD34^+$ cell mixture in which 43% of cells were transduced [177]. In all of these studies, no viral load inhibition was achieved *in vivo.* However, when splenocytes were isolated and sorted into pure fractions of anti-HIV transduced cells, viral inhibition was achieved. In our first set of experiments in humanized mice, we transplanted a mixture of untransduced and transduced cells as described above, and we maintained low initial transduction rates of the $CD34^+$ cells. This low transduction rate was maintained to limit the number of integrated vector copies per cell which has safety implications, and this would allow us to determine whether there would be any antiviral effects with the resulting low frequency of anti-HIV cells in the periphery. Indeed we saw an expansion of the anti-HIV transduced $CD4^+$ T cells in the peripheral blood of these mice, although it must be stated it was a rather limited expansion which did not translate into viral load inhibition *in vivo*. We had expected that in this scenario, there would be a much stronger selection of anti-HIV transduced cells due to the killing of untransduced cells by the

virus, however the continuous renewal of untransduced cells into the periphery coming from the majority of untransduced engrafted human hematopoietic progenitor cells allowed HIV to replicate unhindered. Eventually, a general loss of $CD4^+$ T cells occurred in these animals, as well as an inversion of the normal CD4/CD8 ratio. Similar results were reported when comparable or even higher transduction rates of $CD34^+$ cells were used [56,167,170]. Higher transduction rates on transplanted $CD34^+$ cells resulted in higher frequencies of transduced progeny cells in the periphery that, in some cases, resulted in a transient viral load inhibition, but no long-term effect on viral load was achieved *in vivo* [56,167,170]. Holt et al; showed a significant but short-term viral load inhibition with their strategy [54]. However, it is difficult to compare these results directly since the authors modified the $CD34^+$ cells by nucleofection and not transduction with lentivectors. Kitchen et al; used very low transduction rates and reported some antiviral effect but again short term [57]. Importantly, they mentioned above and others demonstrate complete or substantial HIV viral load inhibition only in *ex vivo* purified transduced splenocytes from humanized mice transplanted with untransduced and transduced $CD34^+$ cells [55,56,59,177]. We also demonstrated that the purified transduced fraction of spleens from our mice transplanted with a mixture of untransduced and transduced $CD34^+$ cells resisted HIV infection *ex vivo.* Importantly, in our experiments, the initial transduction rate of the transplanted $CD34^+$ cells was <30%, demonstrating that our miRNA-mediated CCR5 knock-down is comparable in its ability to inhibit HIV infection to recently published work [55,56,59,177].

HIV gene therapy is more complicated and difficult to cure than other diseases, such as Wiskott Aldrich syndrome. With very few cells and very quickly, HIV can establish a latent reservoir in the mucosal and lymphoid compartments, ensuring its persistence even when HAART treatment is administered [178]. HIV establishes a permanent reservoir extremely early on in infection (i.e., within the first days) [178], and HAART alone cannot deplete the latent reservoir. Gene therapy for HIV cannot deplete the latent reservoir, and any untransduced cells will provide HIV with an opportunity to replicate. So the main hurdle is to achieve high enough

frequencies of transduced anti-HIV progeny cells *in vivo*. Furthermore, HIV can evolve and develop escape mutants, depending on the selective pressure applied. Thus, critical co-factors or viral genes that are targeted must be completely down-regulated or blocked, and coverage of these various targets must be extensive enough to prevent escape. To succeed, any anti-HIV vectors must perform at a very high level. On the other hand, for other diseases which are being treated with gene therapy (e.g., Wiskott Aldrich syndrome), all that is required is some expression of the correct version of a gene that is lacking in a proportion of the cells transplanted back to the patient. This level of transgene expression being sufficient to provide a positive clinical outcome [132,133]. Indeed, in certain diseases, cells expressing the transgene are naturally positively selected due to a survival advantage: the gene in question is directly linked to correct functioning and survival of certain immune cells. [132,133].

With the issue of frequency of anti-HIV transduced cells being crucial for HIV gene therapy, the aim of our second set of *in vivo* experiments was to address this and to try and establish what the threshold is? What proportion of transduced cells is required in order to provide enough protection to inhibit HIV replication *in vivo*? We maintained the use of low transduction rates of $CD34^+$ cells, eliminated the untransduced cells prior to transplantation by FACS and transplanted only anti-HIV transduced cells into the mice. This resulted in mice with practically pure populations of transduced progeny cells in the periphery. Upon infection, viral replication never reached the normal plateau and was significantly inhibited for an extended period of time. Despite the ongoing low levels of viral replication, these animals did not experience the usual HIV-associated loss of $CD4^+$ T cells. Furthermore, expansion of central memory $CD4^+$ T cells, which have been suggested to contribute to enhanced control of HIV infection, was evident. Central memory $CD4^+$ T cells are an indication of immune reconstitution upon HAART treatment [139,143,179]. At this point, we provided the first data showing the generation of mice with pure populations of transduced cells *in vivo* resulting in *in vivo* long-term HIV viral load control. The ongoing low level viral replication observed was most likely due to the engraftment of a

small population of untransduced cells that remained after the sorting prior to transplantation. This population only represented approximately 7% of the total transplanted cells, and again, this illustrates the challenges we face with finding a cure for HIV with gene therapy. The margin for error is very small. Our data indicate that practically all cells must be transduced with an anti-HIV RNA; indeed, even in our model, HIV established itself and replicated. The main effect of our data is the proof of concept: to provide an effective gene therapy cure for HIV, either very effective *ex vivo* selection or *in vivo* selection strategies must be implemented alongside highly effective anti-HIV transgenes.

An aspect that was not examined in this work is whether HAART implemented for a defined period of time would affect the ability of HIV to rebound in an environment made up of primarily anti-HIV transduced cells. Furthermore, these studies were conducted in humanized mice that do not harbour a fully functional human immune system. We can only speculate what degree of protection this frequency of anti-HIV gene-engineered cells could provide in an HIV patient since a patient's immune system can recover significantly under HAART. Perhaps this frequency of anti-HIV engineered cells in a patient could provide sufficient protection to allow the immune system to reconstitute itself and significantly suppress the viral load, this also being the result of possibly a much more efficient selection of the anti-HIV cells by HIV in this setting. This would open up the possibilities for a range of new ways to treat HIV, such as complementing gene therapy with HAART, HAART for limited periods of time, or even more simplified HAART regimens, as well as possibly combining gene therapies with therapeutic vaccines. The major hope for HIV gene therapy is to provide a definitive cure for HIV or at least to alleviate the burden of current HAART regimens in terms of dosage or duration of treatment. This would not only benefit the health of patients worldwide but also address issues of cost and compliance to life-long HAART.

OUTLOOK

1) New anti-HIV targets: Since we have provided the first proof of principle that our CCR5 targeting strategy can provide protection against HIV in humanized mice, our lab has initiated the development of new lentivectors to express further anti-HIV genes to compliment the anti-CCR5 microRNA cassette. These new anti-HIV genes will act as decoy molecules to disrupt the APOBEC-Vif interaction in such a way to increase the amount of anti-viral APOBEC3G. APOBEC3G interferes with the reverse transcription of HIV as well as viral DNA integration. The HIV Vif protein (viral infectivity factor), blocks the antiviral activity of the human APOBEC3G enzyme, this interaction results in the ubiquitination and cellular degradation of APOBEC3G. There are 3 new vectors planned: **i)** Over-expression of a mutant APOBEC3G (D128K) which Vif is unable to bind to due to the point mutation [180,181]. This mutant APOBEC3G is otherwise fully functional. **ii)** Over-expression of a truncated form of the Vif protein which cannot bind to the proteasomal degradation complex and thus should not be able to direct the degradation of APOBEC3G. This truncated Vif will compete with wildtype Vif for binding to APOBEC3G which should result in more APOBEC3G available for incorporation into newly produced viral particles. **iii)** Over-expression of a truncated form of Vif which cannot bind to APOBEC3G but is still able to bind to the proteasomal machinery. Excess of this Vif mutant should saturate the proteasomal machinery and prevent the degradation of endogenous APOBEC3G bound to wildtype Vif. **iv)** The anti-CCR5 microRNA which we called mirGE has performed extremely well. I have however tried to further improve our knockdown capabilities by designing a new microRNA which I have called miR-Extreme. In brief, miR-Extreme has the same 5' flank, lower stem and upper stem configuration as mirGE. The loop has been replaced by smaller 9 nucleotide super loop [182], and the 3' flanking sequence has been replaced by a sequence from a recently described microRNA

called miR-E [183]. miR-Extreme has been designed to target CCR5, it needs to be cloned into an appropriate vector to be tested.

Once all vectors are tested, the final plan is to combine the most effective decoy Vif or APOBEC3G with the anti-CCR5 microRNA genes in a single construct.

2) Selection strategies: I have developed lentivectors that express various genes that provide resistance or in one case increased sensitivity to various drugs. The drugs we have chosen are antifolate drugs used in cancer treatments (methotrexate and trimetrexate) as well as hydroxyurea which is also a cancer drug. These drugs have been or are used routinely in clinics. The reason for developing such vectors is to allow for the *ex vivo* or *in vivo* selection of anti-HIV transduced cells as we believe HIV gene therapy can only work sufficiently well if the vast majority (>90%) of HIV target cells are transduced with an anti-HIV genes. Two vectors have already been constructed and testing is underway **i)** Expression of the human RFC1 gene and RRM2 gene in a single construct. Expression of hRFC1 results in cells being resistant to trimetrexate which means that untransduced cells can be negatively selected, but at the same time cells are more sensitive to methotrexate, so if needed, the transduced cells can be eliminated. In parallel, expression of the RRM2 gene makes cells resistant to hydroxyurea. The possible benefit of expressing two positive selection genes simultaneously is that lower concentrations of each drug could be used if the two drugs have a synergistic effect. This has still to be tested. **ii)** Expression of a mutant form the human dehydrofolate reductase gene ($DHFR^{fs}$). Methotrexate has a very similar structure to folic acid, and has a 100x higher affinity for wildtype DHFR compared to folate. Methotrexate however cannot bind to the mutant $DHFR^{fs}$ gene and therefore cells expressing the $DHFR^{fs}$ gene are resistant to methotrexarte resulting in a powerful selection tool.

Once we have determined which selection strategy is the most efficient, the ultimate goal is to generate a vector with the anti-CCR5 microRNA; a 2nd anti-HIV gene (decoy) as

well as the selection gene or genes. This final vector will be tested in a humanized mouse model and for this phase of testing will most likely include a reporter gene. It might be crucial to test in parallel a second version of this construct without a reporter gene since we plan to use this vector in a Phase I clinical trial.

3) Phase I clinical trial in South Africa: The long term objective is to use the final lentivector which is the product of the work described above to produce lentivector particles and transduce patient $CD34^+$ progenitor cells under GMP conditions. Since the advent of HAART, the frequency of AIDS-Non-Hodgkin's lymphoma (HIV-NHL) has decreased [184]. This has been attributed to the improved immunity and decreased risk of opportunistic infections due to modern HAART regimens [184]. Ironically, worldwide there has been a recent and dramatic increase in the numbers of HIV patients developing non-AIDS defining cancers such as Hodgkin's lymphoma (HL) [185]. In South Africa it seems there is a different trend, there has been a sharp increase in frequency of HIV patients developing HIV-NHL despite HAART as well as an increase in frequency of HIV patients developing HL [157]. Since 2002 the numbers of HIV patients with NHL has increased from 20–30 patients per year to 70–80 patients per year [157]. During the period 1995-2004, 19.5% of HIV patients from the Chris Hani Baragwanath Hospital near Johannesburg presented with HL. However this has now increased to over 50% [157]. Since these patients require chemotherapy, the ultimate goal would be to obtain hematopoietic stem cells from such patients from the peripheral blood following G-CSF stimulation or directly from bone marrow prior to chemotherapy. The stem cells will be transduced *ex vivo* with the anti-HIV-selection lentivector discussed above. The autologous transduced hematopoietic stem cells will be infused back into the patient with the hope that both diseases can be addressed in this way.

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